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STUDIES IN PERIODIC PRECIPITATION

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(WITH FIVE PLATES)

Introduction

Much has been suggested or asserted concerning the significance of periodic precipitation in the interpretation of various biological phenomena, such as markings of surfaces, etc., and it is unnecessary here to do more than mention the names of Liesegang, Leduc, Küster, Klebs (6), Bechhold to recall the discussions following this direction.

But it can hardly be doubted that, valuable as the pioneer work has been, all these suggestions have been tinged, if not deeply dyed, by vagueness or obscurity, so much so as to arouse skepticism as to the value of, or antagonism to the inferences drawn. Nevertheless a residuum of feeling remains that there is something in it all. The stripes of a zebra may be held to be produced in some other way than by periodic precipitation, but attentive consideration of these and similar remarkable productions generally leads to the admission that they look most suspiciously like some of the results of periodic precipitations; and more especially when the irregular or anomalous results of physicochemical experiments are contemplated. Of the latter, some illuminating examples will be given in the proper place.

It is patent that we know so very little about the physical phenomenon itself that the search for analogies is not at present very far justified. This is hardly surprising when we consider that it has been studied chiefly, when seriously, by persons more apt in physical experiment than in biological observation and interpretation. In their singleness of purpose they have sought out the simplest conditions for study, and this from their point of view is right and proper. But biological phenomena are overwhelmingly complex, and it happens that the more complex non-living systems are highly suggestive to the biologist. For this reason is justified their study,

which more than frequently uncovers problems to the physical chemist which otherwise might escape attention. It was the desire to explore this interesting field from the biological point of departure that prompted the authors, aware of both these values, to start on a perhaps perilous course. They emphasize the undeniably extreme complexity of living, and for that matter, non-living biological media, and the fact that it is in these that diffusions and the meetings of substances must take place. Further, unless it can be shown that there has been developed along the ages, means to prevent the interaction of colloids and crystalloids so as to produce periodic precipitations, these must in fact occur, however they may be obscured by the circumstances of their production. It can also be added that, even in non-living systems, patterns may be formed which, in the absence of known conditions of formation, and because of lack of periodic character, would by no means be referred to the proper category of phenomena.

Earlier observation on trichomes

The senior author's attention was drawn to the occurrence of periodic precipitations in the trichomes of plants (*Pelargonium, Saintpaulia*) (**7**) when treated by a method (for the microchemical detection of potassium) which had been tested and discussed by several writers. This consists in subjecting the tissues or cells which are the subject of inquiry to the action of cobalt sodium hexanitrite. After thorough washing in ice cold water for a length of time supposedly sufficient to rid the material of all free cobalt reagent, ammonium sulphide is applied in order to blacken the potassium-cobalt salt, itself pale yellow. It is unfortunate that all cobalt in an adsorbed sodium cobalt complex also blackens under the same conditions, so that there is always a doubt as to the results obtained. This doubt was felt in connection with the testing of trichomes for their potassium content, since, after the above briefly outlined treatment, the advance of the ammonium sulphide along the trichome from the base to apex (or also in the reverse order if the tip of a trichome happened to be broken), produced rhythmic precipitations of remarkable regularity, LLOYD (**7**). ROUPPERT (**11**) has also been able to procure like results in the stinging hairs of *Urtica dioica*. For a review of this field see ROUPPERT (**11**).

The material shows that under the conditions employed, the precipitation lies against the cuticle and wholly within the cellulose wall, not in the lumen. It would appear that the necessary condition for this result is that the cobalt reagent is bound to, or absorbed by the whole cellulose wall, including the septa. The entering reagent diffuses freely along the lumen of the cell but into the wall less readily. The directions of diffusion from lumen to wall give gradients, the form of which must affect the position of

the precipitation, which, lying wholly within the cell wall registers these diffusion gradients. There can be no doubt that the entering reagent diffuses less readily in the wall than in the lumen, for the septa,[1] in trichomes provided with them, cause interruptions in the periodicity, this depending, it would seem, on the altered diffusion rates of both reagents. Since, however, the cobalt reagent is held more firmly by the cell wall, it seems that the effect here is chiefly on the diffusion rate of the ammonium sulphide entering reagent. The continuously steady gradient of periodicity in ROUPPERT's experiments (trichomes of *Urtica*) was due to the absence of transverse septa.

This is not the only irregularity met with. The rhythms in various cells of a series in a trichome may alter from time to time, in contrast to the surprisingly great regularity at other times. In spite of all, however, the periodicity is quite as definite as when occurring in glass tubes (figs. 6, 7) if not as regular (figs. 1–5, plate I). The differences, aside from these irregularities, are referable to the special condition within a trichome, in which the cuticle corresponds to the glass wall, and the cellulose to a lining of gelatine, and not a solid column.

In the several cells of a series in a trichome, however, the periodicity may continue harmonically, relatively at least, disturbed only by the presence of septa.

In many experiments we have made, a periodic system of blue (cobaltous hydroxide) rings was observed, preceding the advancing black precipitate of cobalt sulphide, referable to the concentration of and the more rapid diffusion of NH_3-ions. We observed no blue rings in the senior author's earlier experiments. The occurrence of these blue rings depends on the concentration of the cobalt reagent, since the concentration of the entering reagent $(NH_4)_2S$ was probably too low (about 0.5 per cent.). It was in the form of equal parts of glycerine and $(NH_4)_2S$ of the strength usually used for qualitative work. The internal reagent therefore was probably too concentrated. We must point out that these earlier experiments were purely qualitative, but serve to bring out the following facts: When compared with rings in capillary tubes done at the same time and with similar concentrations of the same reagents, the topography of the rings in the trichomes is seen to be quite peculiar to the conditions therein imposed. In their original form, that is, when first laid down, the rings had a curved outer surface touching the cuticle at the point of original deposition. In the region where the rings are crowded, and where the precipitation took place rapidly, the outer limb of the deposit remained curved (fig. 1,

[1] HAUSMANN (3) showed that the frequency of rings in tubes depended on the viscosity of the gelatin.

LLOYD (7)). Further up the trichome the rings became flatter bands, a greater extent of the outer limb lying in contact with the cuticle. Because of the density of the deposit nothing more than this could then be seen. After standing four years the black precipitate has partially disappeared, the denser portions remaining. One such trichome is reproduced in fig. 1, in which now can be seen the peculiar form of the deposit. Figure 3a was made by enlarging a portion of fig. 1, and fig. 3b from a part of fig. 2. The topography indicated can have no other meaning than that available in the relative densities of the precipitate, this being due to diffusion gradient during formation. During the interval of four years since the original preparation was made, it is probable that S-ions have disappeared through the cuticle, in which they must be soluble to some extent. In another case (fig. 2) the rings were very narrow and sharp, and, as in all cases, but still more evidently, confined to the cell wall[2] and touching on the cuticle.

During the past few months a student, Mr. A. B. A. Evans, has been repeating the senior author's earlier experiments, so far as the reagents could be duplicated. Working with *Pelargonium*, he also obtained periodic precipitations. The character of these precipitations was visibly discontinuous, in some cases quite obviously so. They consisted of minute to coarse maculae, sometimes arranged in longitudinal rows, and apparently departed somewhat from the character of true periodicity. We believe, however, that such aberrations do not invalidate the periodicity (fig. 5), but merely indicate that the conditions obtaining have induced the formation of larger droplets of metastable colloidal precipitate, a condition held important by HEDGES and MYERS (4), and seen by MÖLLER (9). In the other cases (fig. 4) the apparently continuous bands are resolvable, under higher magnification, into a cloud of minute, discrete droplets, so that the difference between the two is one of degree. It is to be noted, however, that the disposition of the precipitate is to a considerable degree controlled by certain markings on the surface of the trichomes, in the form of minute longitudinal tubercles which influence the emplacement of the precipitate. This has been observed by ROUPPERT. It is not mere accident; the same thing happens when for any reason the cuticle happens to become longitudinally folded. In this way the formation of minute capillary spaces favors the formation of periodic precipitation, as ROUPPERT would say. We prefer to say that it is the approximation of surfaces which does this; and this interpretation applies also to the minute tubercles just mentioned.

It seems evident to us that in the case of trichomes a fundamental fact emerges, namely, that the cuticle furnishes a surface or surfaces on which

[2] The bands observed by MÖLLER (9) were also in the wall.

is induced periodic precipitation. Local changes of form of the surfaces, such as pittings (to fit the minute tubercles) and foldings have an added influence in bringing surfaces into closer approximation.

Another easily overlooked feature of the precipitation bands in trichomes is the fact that contraction of the cellulose occurs in these regions due to the dehydrating effect of the precipitate. The trichomes appear then as if cinctured. The degree of contraction is not very obvious macroscopically in photomicrographs but is at once revealed on examining fig. 3b; ROUPPERT's fig. 7 shows it also. We have observed like behavior in the case of cobaltous hydroxide and of cobalt sulphide rings in gelatin.

Earlier observations on capillary spaces in tubes and between glass plates

The senior author made some attempts to reproduce in capillary glass tubes rings similar to those seen in trichomes, but failed for reasons which are now obvious. The tubes were filled with about 15 per cent. gelatin ("Gold label") mixed with a small amount of cobalt reagent. The ammonium sulphide was mixed with an equal amount of glycerine and the tubes were laid in the entering reagent. Two forms of periodic precipitation resulted (figs. 6, 7). The one, in four larger tubes, was characterized by biconvex lenticular discs of very great regularity of form apparently harmonically spaced, alternating with evident but obscure thin rings. In the smaller tubes, instead of regularity, irregularity resulted, not at first, but as the reagent penetrated from both ends toward the middle of the tube. The precipitate consisted in partial rings attached to the wall of the tube, those on the two sides of the tube alternating more or less harmonically. Broad flat bands (discs) are formed toward the end of the process, as the concentration at the middle of the tube becomes exhausted. We have observed this also in cobaltous hydroxide rings, which are discussed later.

There are many details of structure in these tubes which might be commented upon, but as they have stood for a long time, and since during the interval secondary changes due to dissolution of the precipitate have occurred, no further description is given. Original observation, however, disclosed the fact of the great regularity of the periodicity, the lenticular form of the bands and their attachment to the glass wall. The final band formed, at the middle point of the tube (in the larger ones), was always irregular in thickness and placed midway between the last members of the approaching series.

Experiments made by allowing the entering reagents to diffuse into cobalt-gelatin under a cover-glass afforded equally distinct rings traversed by a pointed cross-shaped pattern, having the apices of the arms at the cor-

ners of the cover-glass. This is an invariable accompaniment of diffusion beneath a square cover-glass. The irregularities in precipitation consisted in transition from circular (really only apparently so) into spiral as the center of the cover was approached; branchings (always seen under covers if periodic precipitation occurs); and patterns due to adsorption of precipitate on surfaces not only of the glass enclosure, but of bubbles accidentally occurring. The pattern within the gelatin was not identical with the pattern on the surfaces, a feature which later on we shall see is of great importance.

The presence of many and various irregularities leads us again to emphasize this feature. We have seen that peculiarities of structure in trichomes determine or modify the character of the patterns in periodic precipitations; similarly, the presence and the form of surfaces, even of the wall itself and the surfaces of bubbles, etc., both influence these patterns in tubes and parallel walled capillary spaces. Among these irregularities are spirals (HATSCHEK (**2**)), maculations, disparity of patterns as between precipitate in the body and on the surface of the vehicle, to mention no more. When these facts are considered it becomes easier to expect, but perhaps more difficult to recognize, analogies in the animal and plant.

It becomes evident that, starting with uniform concentrations of reagent in the gelatin, different patterns may arise; and this opens to devastating doubt the whole question of the availability of the cobalt reagent as a test for potassium. It was found (LLOYD (**7**)) that the entrance of the reagent into the cell was marked by membranous precipitations which could not be interpreted as anything more than precipitation patterns. We may now assert, in the light of a reëxamination of earlier observation and of others now to be surveyed, that such precipitations are in reality periodic, and, far from indicating the emplacement of potassium in the cell (MACALLUM (**8**)), show rather that, the cell being a complex of colloidal materials possessing numerous interfaces, patterns which arise from periodic precipitations must occur, and that these patterns may, indeed, be of great complexity.

The previously described experiments with capillary tubes (figs. 6, 7) gave such beautiful periodicity that the precipitations were made the subject of measurement. Diffusion of the entering reagent was from both ends simultaneously. We note the following characteristics displayed by eight such tubes. Four smaller ones gave precipitations which were irregular. The rings were frequently partial, as if broken into halves which were displaced into zigzag positions. If the separate sections could be joined together, a spiral, approximately, would result. The rings (or rather plates) were broad in these four cases, and, aside from the features noted,

were regularly periodical. Of the four (on the left hand in figs. 6, 7) which were very regular, one showed disc-like thickenings in the plates of precipitation, from which had also been built up irregular dendritic processes extending backwards, *i.e.*, toward the nearer opening of the tube or opposite to the direction of diffusion of the entering reagent (the third tube from the left, fig. 6). There are evident also two systems of precipitation figures which are both black at present. One of these two may have originated as cobaltous hydroxide but of this now we cannot be sure. This would be the system of fainter transverse stripes seen in fig. 6 (tubes 1, 2 and 4 from left). The more definite and pronounced system was measured, applying the scale to the projected images from lantern slides, by Mr. A. B. A. Evans. It was found that the results do not support the conclusions of SCHLEUSSNER (**12**), who maintains that the increase in the intervals between rings follows a geometrical progression, but rather harmonize with those of HAUSMANN (**3**).

Bubbles

In experimenting with cobalt sodium hexanitrite we found great difficulty in avoiding the formation of bubbles, and consequently labored under a feeling of frustration until we made a virtue of necessity and used the bubbles to enable us to further our interpretations.

These are bubbles of gas (NO, N_2O and N_2O_3) arising from the decomposition of the reagent, which proceeds more rapidly at higher temperatures (room temperature), in lower concentrations of the reagent, and at lower concentrations of gelatin, all due to lowering of the viscosity. Light also hastens the decomposition and production of bubbles.

When a mass of gelatin is exposed to a reagent, swellings or shrinkings may occur, as is well known. If in the form of a column, and exposed, *e.g.*, to ammonia (as in our experiments), the proximal end of the gelatin column swells and may protrude from the tube (fig. 36, plate III) for various distances. What is not at once apparent is that the middle of the column swells more than the peripheral regions where it sticks to the glass wall. Still less apparent is the fact that this strain is communicated beyond the diffusion limit of the entering reagent to the whole of the column of gelatin. This would have escaped notice but for the fact that bubbles betray the strain and assume corresponding shapes. They often arise in contact with the glass surface and, especially in small tubes, appear attached to it. In consequence of strain they are secondarily stretched and distorted in the direction of the mouth of the tube, assuming beautifully symmetrical shapes (figs. 10, 12, 13). The amount of distortion depends on the position of the bubbles, those in the middle point of the tube (as-

sumed to be open at both ends) remaining spherical. In fig. 10, in which this condition is seen, it is to be noted that the bubbles, because of the amount of distortion, overlap, as seen more clearly in fig. 12.

As may be inferred, the total volume of the bubbles may be very great, and it must be pointed out that the resulting difference in volume of the gelatin is not equal to the volume of gelatin protruding. The total volume of the gelatin is therefore reduced, and internal syneresis must have occurred.

It is also evident that the strain set up at or near the open end of the tube is transmitted throughout the tube. This is demonstrated also by the anisotropy of the gelatin column when suffering the strain. A freshly prepared tube shows none. The bubbles[3] also set up local strains which become evident by the same means.

The syneresis which we have inferred to occur displays itself sometimes by local transverse shrinkage. In one instance the gelatin left the glass wall for a greater part of the periphery between two rings (cobaltous hydroxide). Along spaces thus produced the gas which forms may travel toward the open end of the tube and escape. The evolution of gas becomes greater in the older parts of the column and causes much disturbance. The great variety of surfaces thus induced plays havoc with the regularity of precipitation, due to locally retarded diffusion, and causes most interesting irregular patterns to arise (*e.g.*, fig. 16).

The transmission of strain in a column of gelatin can very easily be seen in a gelatin-cobalt reagent complex in test tubes (fig. 9). Syneresis taking place, the additional strain is in course of time equalized by tearing of the gel. This begins naturally at a bubble and the cavity then becomes enlarged by conchoidal fracture. This is better seen when only a few bubbles are present (fig. 8). By regularly repeated fracture in one direction, long bubbles are obtained which have all the appearance of periodicity (fig. 11). One can see, however, that the tearing is merely a result of regularly repeated spasm caused by steadily increasing strain. It is a special case of conchoidal fracture, such as is readily seen in gelatin when it undergoes internal syneresis (fig. 8). Remarkable examples of such fracturing in gelatin-cane sugar in approximately saturated solution have been procured on a larger scale and we are confident that this behavior cannot be relegated to the realm of true periodicity. The strains, it is true, might result from periodic alterations in water content, but of this we have no certain knowledge.

[3] The gas in the bubbles is under a low pressure, for if a part of a column of gelatin containing bubbles is removed, the thinner wall, if external, collapses. If now the bubble be punctured under fluid, this rushes in and a smaller bubble remains inclosed in the fluid which has entered.

When conchoidal fracture intervenes there is usually evidence of twisted strain, in consequence of which a bubble may twist on its axis during growth and may even become definitely spiral. One here wonders how far strains in gelatin may play a part in producing the spiral precipitations noted by HATSCHEK (*l.c.*).

The presence of a bubble occupying a large portion of the cross section of the tube has disturbing effects on the direction and rates of diffusion. There occur consequently displacements of periodic precipitations resulting in twisted, broken and otherwise altered rings or bands. These will better be considered further on, it remaining now only to reiterate the importance of the surfaces introduced by bubbles. We find that the surface of a bubble lying in the path of diffusion affords the place for the accumulation of precipitate as part of the periodic system produced; while the bubble as a whole, being an impediment in the diffusion path, diverts the diffusion currents so that stream lines are set up analogous to those in air or water caused by relative motion of a body.

Periodic precipitations of cobalt sulphide (CoS)

When cobalt sodium hexanitrite is mixed with gelatin and placed in contact with ammonium sulphide, a black precipitate of cobalt sulphide is formed. This forms a barrier in the gelatin through which ammonia permeates more rapidly than S-ions. In consequence, when tubes, etc., are loaded with the cobalt-gel complex, the ammonia of the entering reagent moves forward more rapidly and produces in advance of the black periodic precipitation of CoS, a periodic system of blue rings of basic $Co(OH)_2$ if the concentrations are high enough. We shall consider the sulphide precipitate first, but must speak of blue rings at the same time. We leave more detailed consideration of the latter till later.

We have studied the character of the periodicity in the black precipitate (as we shall hereafter designate the CoS) in capillary, medium sized, and larger tubes. The inside diameters of the tubes were 0.37, 0.54 and 0.57 mm. for the small, 3.62 mm. (two long tubes), 2.96–3.40 mm. for a quartette of tubes, 7.1 for another, and test tubes 12 mm.

During the earlier period of diffusion, the precipitate formed is for some time apparently homogeneous (figs. 14, 16). It is usual to say of this condition that no rings have been formed, and the inference is that periodicity has not as yet occurred. This, however, is not the case, since if the column swells and protrudes from the end of the tube, the rings or bands become apparent (fig. 36). The periodicity in the tubes in fig. 6 is visible, at the outer ends, only under the microscope. The failure to produce even microscopically visible periodicity is, it seems, no proof that periodicity is absent.

Again, after long standing, secondary changes occur in the black precipitate which betray the periodic structure. Before any periodicity becomes obvious, examination by reflected light properly adjusted will reveal to the eye the zonation on the inner glass surface (fig. 32). Later the zones become quite distinct (fig. 33) until they become separated into groups of rings. The outer end of the gelatin column, which projects into the vessel containing the entering reagent, at length breaks up into these groups, being set free by the dissolution of the binding gelatin (figs. 64, 69, plate V). By study of portions of the column which have lain in ammonia for some time (fig. 69), we can see quite clearly the composition of the system, which is obscure and only partly decipherable when still in the tube. Before revealing the structure so seen, we must examine the periodic precipitation arising from the more rapid entrance of the NH_4-ions.

Ahead of the front of the black column arise blue rings and discs, the Saturn structure of Popp (**10**). In positions where these are sufficiently separate for observation it may be seen that the black column gradually advances beyond, let us say, a given blue ring. At about this time the next blue ring, which is later to be overtaken gradually darkens. After being overtaken it continues to darken till it becomes dead black, so that it can be recognized within the black column by its contrast in this regard (figs. 32, 33 to be compared with fig. 21). The cobalt sulphide precipitate is relatively grayish and can be recorded photographically. As the column advances, or also with long standing, the black column included between each two blue rings becomes resolvable into several rings. The number of rings at first appears to be determined wholly by the numbers and positions of the blue rings, there seeming to be a necessary relation between the two sets of periodic precipitations. In order to test this we produced a periodic system in gel-cobalt sodium hexanitrite, with ammonia as the entering reagent (fig. 39, plate III). After the precipitation was complete, we substituted ammonium sulphide for ammonia upon which we observed the dissolution of the blue rings as they were overtaken by a deeper brown *clear* zone, considerably in advance of the front of the black precipitate.[4]

The rings in the black had a much higher frequency and bore no relation numerically or spacially to the erstwhile blue rings. There is, therefore, no necessary relation between the two systems. In the experiments recorded in figs. 14–22, plate I, and 24–31, in plate II, however, the conditions were such that the blue rings never disappeared. They merely blackened and later retained their identity, so much so that they were found intact in the vessel containing the ammonium sulphide entering reagent several

[4] As to the chemical nature of this clear zone, we cannot at the moment decide. It is perhaps to be considered as red $Co(OH)_2$ formed in a higher concentration of newly entering ammonia from the $(NH_4)_2S$.

days after the swollen column had been expelled from its tube. Pieces of the gelatin column containing the two systems of rings thus obtained (fig. 69) were gently heated to see if the gelatin had been altered in regard to its softening point. We found that the black rings dissolved at once, but the blue rings remained intact (fig. 69). The color of these intact rings in strong light was distinctly bluish, though casually to the eye appearing black. We interpret this as indicating the adsorption of the black on the blue salt. A certain disturbance of the spacial arrangement of the black rings, due to the previous presence of the blue, has been observed; and this would, if adsorption occurs as indicated, be inevitable. Such delicate rings are very fragile and may be sustained merely by the continuity of precipitate and not by the gelatin. We should here note that the blue rings shrink shortly after formation (fig. 22), and there is no doubt that the shrinkage results from a dehydrating effect on the gelatin; this so contracted zone remains relatively incapable of responding to the swelling effect of ammonia.

The black rings have a ring-and-disc structure (fig. 66), precisely that "Saturn-structure" described for blue cobaltous hydroxide by POPP (**10**). We found this to be true also of the precipitation (fig. 38), when procured in large tubes (fig. 35).

When first laid down, the black precipitate appears first in banded form, but the position of visible bands is not constant. In no case have we been able to observe the same number of discrete bands as subsequently become visible; these usually appear to the number of one or two, while subsequently five or six appear (fig. 69). Later, on standing, whitening of some rings takes place (fig. 32). This may be due to a lower cobalt sulphide content or to secondary changes or changes by condensation toward the middle of the rings and dilution of CoS in the spaces between. This distinction disappears with time and we then find uniform black bands of ring-and-disc structure.

Curious irregularities occur. Partial discs are common, and we have found remarkable dendritic or vermiform black masses of precipitate traversing the bands more or less longitudinally with respect to the tube.

After the black precipitate is laid down, a condensation of the gelatin involved occurs as in the case of cobaltous hydroxide, but to a lesser degree. On subsequent swelling in the presence of ammonia the zones of gelatin containing the precipitate do not swell as much as the unoccupied gelatin, so that the freed column shows constriction at the black bands (fig. 69 when examined with a lens). This we have already mentioned as occurring in trichomes, as ROUPPERT also observed. This shrinkage is visible in the tube and becomes so because the total effect of black zone shrinkage is to pull the gelatin away from the tube wall, thus causing reflecting surfaces.

Blue rings

Three large tubes (12 mm. diameter) were charged with 10 per cent. "Difco" gelatin, and 1 per cent. $Co(NO_3)_2$ with 21 per cent. ammonia diluted 1:5, 1:10 and 1:20 as entering reagent. Constant temperature was maintained at 11° C. (fig. 40).

For several days, although periodicity became evident, it lacked regularity. Bands were to be seen but they were sometimes vague, and when more distant, showed no harmonic space relations. During this time the advance of the front of the column proceeded at the following relative rates, the last column being the record at the end of four days.

a (1:20)	19	26	38
b (1:10)	24	32	46
c (1:5)	27	37	54

That is, the rates vary with the concentration but not in constant proportion. After several days, distinct and harmonically spaced rings appeared, earliest in the lowest concentration of entering reagent. Meantime the uppermost zone of the gel became colored red (red cobalt hydroxide) the entering reagent dissolving the earliest formed blue precipitate. In this red region there is no visible periodicity. The extent of this reaction varied with the concentration of entering reagent. The distinct bands had the ring-and-disc structure, "Saturn structure" mentioned by HATSCHEK, described by POPP (**10**) (figs. 66–68). That is, a thin ring or band is adsorbed at the gelatin-glass interface, the disc being a circular plate laid down within the column of gelatin in approximately the plane[5] of the ring. It is difficult to separate the band from the glass. We succeeded in separating a portion of the column and obtained longitudinal sections which display the Saturn structure. It is suggested by these figures (67, 68) that the gap between the ring and disc becomes secondarily filled by the spreading of the precipitate.

The ring-and-disc structure was obtained in beautifully regular form in two smaller tubes (figs. 14–21) 2.25 mm. inside diameter, filled with "Difco" gelatin 10 per cent. and 5 per cent. cobalt reagent (fresh) with aqueous ammonium sulphide 1:10 as entering reagent. Both ends of one tube and one end of the other gave like results. Having been unable at this time to repeat this experiment with similar results,[6] we applied entering reagent to the remaining end, which had been kept sealed by a rubber tube,

[5] We are at the present writing able to state that in general the disc lies in front of the equator of the ring, though whether or not this is true of the earliest ones formed is uncertain.

[6] This has now been done; see later.

and obtained similar, but not, in time, quite identical results. A possible explanation lies in the physical changes (probably syneresis) which had overtaken the complex during the interval.

In these experiments the advancing black precipitate was preceded, as always, by a broad zone of clear yellow color indicating migration of cobalt ions from this region, within which are laid down rings and discs with great regularity so long as the homogenity of the medium (gelatin) remains undisturbed.

During the earlier period, the periodicity is scarcely observable; however, it is doubtful that the first precipitate is truly continuous but is rather only apparently so. Soon, however, a denser central and peripheral region become visible which gives place to distinct periodicity, and each band is then composed of a ring and disc. These may be laid down simultaneously (so far as observation will permit us to say) or, as a matter of undoubtedly correct observation, the ring is sometimes at least laid down *before* the disc (fig. 19).

As the precipitation column develops, the discs become smaller until they are no longer formed; or, as may occur, they may be formed anew to the number of one or two, and sometimes we find small granule-like points or larger discs formed. The presence of a bubble may cause such irregularities to occur, and the disturbance so caused may lead to the formation of incomplete discs (fig. 14) but it may also have no obvious cause. Discs, for this or other reasons, may have perforations or embayments (figs. 64, 70) or they may, less frequently, become connected with the ring by an isthmus. Similarly the discs are not equally thick, nodules or fenestrations occurring. All of these facts must have a bearing on any explanation of the phenomenon of periodicity. We did not find it easy to duplicate the ring-and-disc structure but succeeded finally by using 20 per cent. gelatin with 0.5 per cent. $Co(NO_3)_2$ in tubes 2 mm. in diameter with ammonia 1 : 10 as entering reagent. Within an hour the ring-and-disc structure was visible. The blue precipitate was preceded by a pink zone 1 mm. thick. Beyond this could be seen, by careful illumination, the well-defined face of the diffusion column, quite colorless, and made visible photographically by adjustment of black and white background.

When the tubes used were of capillary dimensions (0.54–0.56 mm.) we obtained only rings, or occasionally plates arising by extension of the ring across the lumen (fig. 37). The bridging of the columns of gelatin may therefore take place by fusion of disc and ring or by thickening of the ring alone. These facts cannot be contemplated without realizing the importance of the glass-gelatin interface in furnishing a footing of fixation for the precipitate in our experiments. The rôle of bubbles in this regard and

in varying the topography of the patterns formed has already been discussed. We were therefore led to test further the possible effect of surfaces on pattern by making up a cobalt-gelatin mixture, introducing thereinto (a) a glass rod running approximately along the axis of the gelatin column; and (b) a lot of glass fragments. The different ends of the tubes were exposed to ammonia and ammonium sulphide. It was difficult to observe what was happening to the rod while in position; that is, difficult to separate reality from reflection. But upon removing the tube after sufficient time had elapsed we found both blue and black precipitates on the surface of the rod (fig. 60). The patterns formed were quite bizarre, and would not, we believe, be regarded as the product of periodic precipitations. While we are justified in regarding the pattern on the rod to be correlated with that on the tube wall, it may be that the forces at play, while strong enough to start the precipitation, were insufficient to locate it in positions of strict correspondence. The patterns on the wall of the tube also showed much irregularity (figs. 58, 59), more indeed than would be expected, but while this may be connected with the presence of the glass rod, it seems unlikely.

In the presence of glass fragments, rings of fair regularity were formed on the tube wall (fig. 62). Less internal precipitation occurred than was expected, but when it did, it always originated on the surfaces of glass fragments and secondarily bridged over from one to the other (fig. 63).

Again, the question of the character of the surface has arisen in the past. We found that the behavior of these reagents remained indifferent to the difference of surface as between quartz ("Vitreosil") and glass.

Just as, in the cases before us, surfaces play a part in fixing periodic precipitations, so the displacements of the medium by bodies affording surfaces provide conditions for the unequal emplacement of reagents, and for changes in the directions of diffusion. In consequence, the periodic bands become displaced, and various twistings and changes in their dimensions take place. The rings seen at and above the bubble in fig. 21 are a clear example. The effective concentration of reagents was attained at an abnormal position, nearer to the last formed ring than otherwise would have occurred. The next formed ring was twisted, and the second following had a gap. The part of this ring that should have occupied the gap was formed further along the tube in a position corresponding to the oblique displacements in the preceding rings. It is evident that the bubble had caused an oblique redirection of diffusion.

Similarly, the dimensions of rings are affected by directions and relative rates of diffusion. In a homogeneous medium (as much as we questionably suppose gelatin to be) the farther the entering reagent has to travel, or, the greater the effective lowering of concentrations, the wider the rings

become (fig. 65). A sufficiently large bubble suitably placed will interfere with the diffusion of the reagents so that an unexpectedly broad ring will occur. If the bubble nearly blocks the column symmetrically the ring occurs at or rather just beyond the zone of smallest cross section (fig. 65). It is probable that such a bubble affects the positions of all the rings of the system.

The width of the rings, in addition to varying uniformly, may vary in any particular case. An individual ring may thus become nodular, and nodes corresponding in position may occur on neighboring rings (figs. 24, 26, plate II). If close enough together these nodes become confluent and a network of square meshes occurs (fig. 23a). Sometimes the transverse elements are so far suppressed that only nodes and their longitudinal connections ever occur. Thus, instead of rings, longitudinal lines of precipitate result (fig. 24). All sorts of intermediate confusions of pattern arise from these aberrations, and the consequent patterns, *e.g.*, fig. 16, are very suggestive to the biologist; and to the physical chemist are examples of periodic precipitation in which the resultant pattern often not in the least reflects the periodicity.

We have already pointed out that the rings are laid down first at the gelatin-glass interface. The ring is at first a flat band or ribbon but in a short time it begins to contract and becomes furrowed (fig. 22). Now it can be seen attached to the glass along two fine lines, the margins of the band. We have sometimes thought that the whole of the gelatin column shrinks, but whichever view is correct, the fact remains that the most complete attachment to the glass is usually along the two margins of the band (fig. 22). In any case the shrinkage of the gelatin appears to result from the presence of the precipitate. Displacements of a ring or other conditions may occur in such fashion that only one margin or a thin zone of the ring is attached to the glass. This we have seen in the presence of a bubble, and it can be seen in the region of the rather crowded broad rings in fig. 26.

The constitution of blue rings appears not always uniform. We have observed that, under some circumstances, blue precipitate is first laid down, followed by white precipitate as seen in fig. 23, which was from a photograph taken through a blue-absorbing filter. Here one sees a double white line bounding the photographically dark (= blue) ring. The white precipitate is probably a form of cobaltous hydroxide, in a fine state of dispersion and lower concentration but the use of the blue-absorbing filter shows that the white color is due merely to the state of dispersal of blue precipitate. These rings ultimately turn blue.

The cobaltous hydroxide and cobalt sulphide precipitates appear far more homogeneous and continuous than others we have observed, *e.g.*, silver

chromate. Examined ultramicroscopically, the former has much the optical appearance of a casein coagulum, and cannot at least be regarded except as a deposit of finely divided, probably metastable precipitate. The latter behaves, as we observed in preparations between glass plates, as other metastable precipitates, *e.g.*, sulphur, in which the smaller droplets disappear and contribute to the growth of the larger ones, which eventually crystallize. We thus obtained crystals of cobalt salts in some, and of sulphur in other preparations.

Although we observed transverse shrinkage in the gelatin column, we recall that the presence of the entering reagent (ammonia or ammonium sulphide) causes the proximal region of the gelatin column to swell. The amount of swelling is greatest along the axis of the column. Radially directed pressure must also exist, as appears from the swelling of the column on leaving the tube (fig. 36). If periodic precipitations are being laid down during the swelling, and the transmission of the strains due thereto are transmitted at a rate to overtake the bands (rings and discs) already laid down, the discs especially become displaced in the direction of the swelling, and are then dished (figs. 20–21). Misplacement may occur because of the presence of a bubble, that is, the disc may appear out of position either as a result of swelling, or from disturbances of diffusion gradients (figs. 17, 18). The strains set up by swelling can be seen, as above stated, between crossed nicols.

White rings

Pale white periodic precipitations of a fine, widely dispersed precipitate were first observed in a test tube holding gelatin only, with ammonium sulphide as entering reagent. The precipitate upon magnification was seen to be composed of minute dumb-bell-shaped, crystalline pairs. As crystallization advanced a second pair might arise at right angles to the original system, now forming a sort of cross (fig. 45). With later growth compound sphaerocrystalline masses resulted. They were determined to be sulphur crystals. This peculiar morphological form arises in gelatin—that is, in a watery colloidal medium—though it is not the only crystalline form so to arise.

Rhythmic crystallization of sulphur in this wise was obtained in the following experiments:

(a). A gelatin-ammonium sulphide mixture was placed in a series of tubes of various diameters and left open. The control was sealed. In all the open tubes periodic precipitation of sulphur was obtained. The figure (fig. 41, natural size) was made before a final ring was added but the preparation suffered from overheating in a horizontal position and so the

photograph of this is not reproduced. The periodicity of the rings was the same for all tubes. The precipitate was laid down as rings close to and in contact with the glass wall, and later filled, thus becoming a plate. The character of the precipitate made it difficult to see if the ring-and-disc structure obtained, though there seemed to be a concentration on the glass.

This precipitation may be the result of one of the following conditions:

(1) Diffusion of oxygen and the oxidation of sulphide to polysulphide and sulphur.

(2) Evaporation of ammonia, leaving H_2S and following the process mentioned in (1).

(3) Dissociation of sulphur from the protein by alkaline hydrolysis. Although white bands are made in like amounts in the tube whether sulphide is present or not in the entering reagent, in both cases it is due to the same cause, since, at the time of its formation in the tubes provided with $(NH_4)_2S$, it always occurs *beyond the limit of the black column,* and hence at a place to which the sulphide has not yet diffused. At high concentrations of ammonia the white precipitate remains longer than at low. In view of the studies of HOFFMAN (5) and GORTNER it seems probable that alkaline hydrolysis is responsible, though we note it to occur at room temperature. Further work is needed to decide this question.

(b). A gelatin-cobalt reagent complex (20 per cent. "Difco" gelatin, 0.5 per cent. cobalt reagent) with ammonia 1:50 as entering reagent, gave no precipitate. Only two successive darker yellow moving bands occurred. On substituting stronger ammonia (1:10) in the course of a week three rings of white precipitate, later followed by three additional rings, six in all (fig. 41, taken before the last ring was formed) were produced in the course of two weeks. The precipitate (fig. 45) was identical with that observed when sulphide was present (fig. 44). Since there was no source of sulphur present in the reagents, we must suppose that some part of the gelatin molecule was split off by the NH_4OH and sulphur set free. The same result was repeatedly obtained. As in the previous experiment the form of the crystal indicates an originally very finely dispersed metastable condition of the precipitate.

Effects of temperature on the above described periodic precipitations

BEHAVIOR AT LOW TEMPERATURE

Four tubes were prepared with gelatin-cobalt complex (20 per cent. "Difco" gelatin; 5 per cent. cobalt reagent). One end of each of the tubes of a pair was exposed to ammonia 1:5, the other to ammonia 1:20, and the

other end to ammonium sulphide 1:5 and 1:20 respectively. One pair of tubes thus prepared was kept for 100 hours at 3° C., another pair at room temperature. The following observations were made (figs. 24–31, plate II).

Diffusion in the cold is not as rapid as in the higher temperature. This may be due, in part at least, to the higher viscosity of the gelatin at lower temperature.

The lower temperature favors the formation of a white precipitate of metastable sulphur, yielding double sphaero-crystals such as have been described previously. If sulphur is present in the entering reagent, white precipitate is found in advance of the black and moves forward with time (figs. 27, 31). It finally becomes evidently periodic.

If only ammonia is present in the entering reagent a second general zone of white precipitate occurs near the mouth of the tube. Two periodic zones occur at lower and 3–4 in the higher concentration. These do not move forward (figs. 25, 29). After 100 hours at 3° C. this pair remained at room temperature, after which the inner zone of white precipitate gradually disappeared and blue cobaltous hydroxide appeared in the same position. At the lower concentration of entering reagent the blue rings (about three in number) are broad, vague and rather irregular (figs. 25, 27); at higher concentration they are more numerous and more sharply defined (figs. 29, 31). The white rings near the mouth of the tubes remained unaltered for a long time, and no blue rings appeared here.

Behavior at room temperature

The behavior of black and of blue rings at room temperature (figs. 24, 26, 28, 30) was in general as already described, except that at the concentrations used there were no discs formed, but rings only. These were wider and vague at low concentrations of entering reagent, and more numerous and more sharply defined at higher concentrations, at which also the distance to which the ring formation was carried on is greater. This distance varies with, but is not constantly proportional to, the concentration of the entering reagent. We note, however, an exception in tube no. 6, fig. 24, which we cannot explain.

Although NH_3-ions must have diffused to the position indicated by the white zone to the right of tube 3, fig. 29, no cobalt rings had been formed. Only as this white zone began to disappear did ring formation occur, and indeed after the tube had been brought into the warmer temperature a single delicate blue ring developed, followed at a distance by others. We see therefore that the entering reagent may move forward without causing periodic precipitation (*cf.* tubes 4 in figs. 28 and 29). This means that a

precise concentration of the reagents is required to cause the precipitate to form in a particular position, neither higher nor lower concentrations sufficing.

Experiments in which the reagents meet in a capillary space between glass plates

The gelatin-cobalt complex was placed between a slide and square cover-glass convenient for microscopic study. The preparation was laid in a petri-dish, and covered with entering reagent which could then diffuse from all sides of the cover-glass.

There was always observed in such preparations a diffusion pattern in the form of a cross with pointed arms, the points at the corners of the cover-glass (figs. 46–48, 54, 55). This "diffusion" cross demonstrates the movement of the reagent in the gelatin to meet the entering reagent. The dimensions of this cross are determined by the relative diffusion speed of the ions involved.

Evident periodicity may or may not appear (figs. 46–55, plates III–IV). While the exact concentration which will produce it is not exactly easy to state we find in general that too high concentration of either reagent was unfavorable.

The results depend also upon the kind of gelatin used; we obtained no visible periodicity till we began to use "Difco" gelatin, although in earlier experiments the senior author obtained rings in all experiments done with "Gold label" gelatin. We think also that the viscosity of the gelatin plays a part, for we found that a long period of setting in a cool temperature, which rendered the viscosity higher and diffusion slower, favored the occurrence of visible periodicity. This is shown also by the fact that, if the entrance of sulphide was preceded by ammonia, as evidenced by the formation of blue rings (fig. 54), the progress of the black precipitation (fig. 55) was more rapid. An experiment kept at first at a lower temperature in a refrigerator, and only a short time at room temperature, gave no blue rings, and a less well marked periodicity of black precipitate (fig. 46).

We have already discussed the relation of blue and black rings, showing that there is generally no relation between the appearance of the periodicities. In our cover-glass experiments, however, we observed exceptions to this, *e.g.*, in the experimental results shown in figs. 54–55, we find that an entire system of blue rings was first formed beyond the advancing edge of the black precipitate and that the pattern later formed by the black precipitate was identical with it. The absence of further black precipitate was due to low concentration of S-ions. We watched the progress of the black precipitate under magnification and found that the blackening of the

blue rings was the first step in the process, the building up of the black precipitate then extending backwards. The significance of this earlier blackening of the blue rings (common, as seen by all our experiments in which blue rings were formed ahead of the black) lies, we believe, in surface relations. The cobalt hydroxide already laid down offers an extensive surface for adsorption of the black precipitate. We shall show that the glass surface, in the absence of the blue precipitate, becomes of paramount importance.

It is not surprising, in view of these facts, that visible periodicity became, in our experiments, very well marked if we either acidified the cobalt (acid) reagent or rendered more alkaline the ammonium sulphide. Such results as are shown in figs. 48–50 were obtained.

The rings were very closely packed. In addition the experiments show the effect of the diffusion of Co reagent from the cross region, leaving here lower concentration. This is reflected in the reduced number of rings per unit distance (measured normally to the rings). The total distance occupied by the periodic precipitation measured along the diagonal of the square is greater than the distance normal to the rings where they are parallel to the side of the square. The total number of rings along the diagonal is greater than elsewhere, and this explains the articulation of rings along the lines of delimitation of the cross pattern.

The pattern of the rings is a function of the surface of the glass plates, and is relatively independent of the concentration (illustrated by the cross pattern, which expresses concentration). We thus obtained two patterns superimposed on each other, the cross pattern and that of the advancing rings. Fig. 48 shows well the patterns in question.

A proper consideration of this principle enables us to interpret the patterns arising when additional variously formed surfaces intervene in the path of a diffusing reagent, as, *e.g.*, when bubbles so occur. The simplest condition to contemplate is that of a spherical bubble (fig. 49).

Furthermore we have observed that always in the case of bubbles the surface plays the same rôle as the surface of a glass tube, *i.e.*, there is always on the surface a greater amount of precipitate corresponding to a band more or less removed. We thus encounter again the ring-and-disc structure (figs. 49, 51, 52, 53).

With respect to the ring between glass and cover, we must say the same; comparing the space between to the lumen of a tube, the distance acts correspondingly to the diameter of tube. If this distance is great enough we shall have a ring (or ribbon) and disc; if narrow, the ring alone may fill the space as in capillary tubes.

In order to test the effect of various kinds of surfaces, we introduced gypsum (figs. 51, 52) and oil (fig. 57). The gypsum reacts with ammo-

nium sulphide, forming calcium polysulphides. These react with the surrounding gelatin, which is thus rendered highly viscous and independent of the general mass; it then has the appearance of a yellowish vitreous mass, containing many irregularly scattered, partly dissolved gypsum granules. The surface of the vitreous masses served for the diffusion of periodic black precipitate, as also the smaller irregular granular appearing masses included. The resulting patterns, even when very irregular, yield to analysis in terms of ring-and-disc structure. This is quite obvious in fig. 51. However bizarre the patterns may be, we see no reason for doubting their periodic nature.

In the case of oil drops we observed an additional matter of interest, *viz.*: the crystallization of sulphur from the metastable black precipitate. These crystals were of metastable forms in the precipitate remaining (fig. 57).

Another feature of pattern is to be seen in figs. 53 and 56, in which the surface of gas bubbles is apparently folded and densely packed with black precipitate. It would here seem that the surface layer of the gelatin in contact with gas space is swollen, the effect of the ammonia, probably. The folding is purely a mechanical adjustment of the surface but we have seen evidence that the swellings are periodical in that they correspond to a periodic precipitation.

The importance of surface having impressed us, we have repeated ROUPPERT's experiment of using fluid cobalt reagent under a cover and allowing the sulphide to diffuse with it. He obtained visible periodicity, between which, however, and the repeated breaking of early formed precipitation membranes (ROUPPERT), we see no connection. We confirm ROUPPERT in his observation of periodic precipitation but we found that almost *identical patterns occurred on the two opposed glass surfaces,* the one looking through the microscope as if it were a "ghost" or reflection of the other (fig. 71). In order to be certain of our observation, we made a second preparation, washed away the reagent and removed the cover-glass; then we found almost identical periodic patterns on the two surfaces. Fig. 72 is the pattern photographed during the progress of precipitation. Fig. 74 is of the slide and fig. 73 of the corresponding corner of the cover-glass. We could watch the formation of the precipitate and the building up of the pattern.

Within the general area of the new ring one can observe the apotheosis of minute but visible granules which were free in the fluid as evidenced by their Brownian movement. Meanwhile a pale bluish zone was being built up on the glass surfaces. It seems that many much smaller and invisible particles of precipitate are being formed simultaneously. These must be-

come quickly adherent to the glass, since the larger visible particles suddenly stop their movement. This occurs evidently at the moment of becoming fixed to the surface.

That this precipitate is in a metastable condition is shown by the fact that clear circular areas are formed immediately when the layer is thin, these areas being caused by the evaporation of precipitate to form small crystals (fig. 71). This has been repeatedly observed in many preparations.

The peculiar pattern seen in fig. 72, where it appears scalloped, is due to disturbance of diffusion. These disturbances probably cause more or less local mass movement, unavoidable under the circumstances of the experiment.

Methods

The observations here presented were made in connection with experiments the chief purpose of which was orientation. The systems employed are admittedly complex.

The periodicities were studied (a) in glass or vitreosil tubes of various bore from 15 mm. to 0.5 mm. As we found no difference in the results obtained with glass and quartz, we have avoided further reference to this comparison; (b) in capillary spaces between glass plates (slides and square cover-glasses).

"Gold label" or "Difco" gelatin was used. The "reactant" was mixed with the gelatin and the mixture allowed to set, after which the entering reagent was added.

Smaller tubes were filled and the ends exposed to the entering reagent in a test-tube opened on the side, the tube being supported in position by a rubber stopper. The two ends of an entirely filled tube, if of sufficient length, could be thus exposed to different kinds or concentrations of entering reagent.

Glass plate preparations were exposed to the entering reagent in petri dishes, covered.

Photographic records were kept. Tubes were surrounded with water or glycerine to obviate reflections. Color screens were used to procure contrast. We find that a photographic record is of the greatest possible use in studying the protocols, as well as in illustrating the observed facts.

Summary

The banded precipitate of cobalt sulphide occurring in the trichomes discussed in this paper lies in contact with the cuticle and within the cell membrane. On comparison with precipitation in gelatin in small tubes, the walls of the tube—or the interface gelatin/glass—is found to correspond

with the cellulose/cuticle interface. Periodic precipitation may occur in the lumen of trichomes, but we have nothing further to say of these at this time.

The precipitate is laid down as fine or coarser granulations or droplets, doubtless in a metastable condition. These granulations become distinctly visible when there is a structural basis, such as minute tubercles, etc. This is due to the approximation of surfaces favoring local adsorption of the precipitate.

The explanation of the localization of precipitate was made clear by our subsequent experiments, in which we found that the surface of the inclosing vessel (the gelatin or water/glass wall interface) plays an important rôle in the emplacement of the precipitate. Thus arises (in all cases observed by us) a ring-and-disc structure, though this is less obviously the case when the precipitate is markedly discontinuous (*e.g.*, sulphur). It is appropriate to mention in this connection that R. B. PEEL, while a student with Professor STEWART YOUNG, found evidence of the adhesion of ammonium chloride in bands on the glass wall of the vessel in which the reaction of ammonia and hydrochloric acid had proceeded. This reaction occurs rhythmically. He speaks of the "catalytic action" of the wall of the vessel (in MS. Library, Stanford University). Further study of such precipitates is desired. It was found, accordingly, that the periodic precipitate of cobalt sulphide in the absence of a colloidal medium, observed by ROUPPERT, resides on the glass surfaces bounding the capillary space. It is concluded that the emplacement of the precipitation is made possible by the nearness of these surfaces. In minute glass tubes the precipitation occurs attached as a ring to the walls, without the disc, for the occurrence of which a tube having a diameter greater than a certain critical value is required. This distance is related to the rate of movement of the ions concerned; that is to say, periodic precipitation does not depend for its occurrence on a capillary space, but its fixation depends on the presence of appropriate surfaces. Thus, in a wide enough tube, and in the case, *e.g.*, of cobaltous hydroxide precipitated in gelatin, the precipitate is adsorbed on the glass wall (as a ring) and within or upon the gelatin itself (as a disc). In the absence of a glass (or other) wall only diffusion-shell patterns would arise, as in the classical OSTWALD experiment. It is urged, however, that some surfaces ("nuclei") are required further. That periodicity occurs in a fully fluid medium (water) does not invalidate this conception.

In insisting on the importance of surfaces we approach the position of BRADFORD (**1**), who believes that adsorption plays an important rôle. The precipitate, he says, furnishes surfaces toward which the surrounding solute

moves, adding to each particle more material. Without pausing here to consider how, according to this author, periodicity consequently arises, we have to say that, the precipitate having once been established, in a particulate condition, the evidence we have seen supports BRADFORD's ideas. The provision of such particles appears to demand a condition of supersaturation (OSTWALD).

The more surfaces that are provided and the more irregular they are, the more diverse the patterns are which arise, nevertheless, from periodic precipitation. We have been led to see that the heterogeneity of the medium, such as the living cell or body presents, must furnish conditions in which extreme irregularity of pattern would arise from periodic precipitations. We should examine biological material therefore, free, at any rate from prejudice arising out of the contemplation of those beautifully regular patterns (the "LIESEGANG rings") which have engrossed the attention of biologists to the exclusion of many other possibilities.

Differences of temperature have been found to affect the results profoundly. Low temperatures did not favor regularity, or even the obvious occurrence, of periodic precipitation.

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Description of figures

Fig. 1. Trichome (*Saintpaulia* sp.) treated with cobalt sodium hexanitrite, followed, after washing, by ammonium sulphide in 1923 (Lloyd, (7)). The black precipitate has to some extent disappeared (by 1927) leaving only the denser portions.

Fig. 2. Another example, in which only the densest zone of each ring is left. At the level of each ring the cuticle is sharply contracted. (See fig. 3b.)

Fig. 3a. A portion of fig. 1 enlarged.

Fig. 3b. A portion of fig. 2 enlarged.

Fig. 4. Trichomes of *Pelargonium* sp. showing periodic precipitation with irregularity of position, probably the effect of the septa. The precipitate occurs as a fine granulation.

Fig. 5. A trichome of *Pelargonium* sp. with minute tubercles on the surface, underneath which masses of precipitate occur. But the grouping is periodic.

Fig. 6. Normal, and fig. 7 oblique, view of a group of capillary tubes (experiment of 1923) showing periodic precipitation in the form of plates or partial plates. A second system, the nature of which is unknown, is visible as faint bands between the more widely spaced dark bands. Tubes contained gelatin-cobalt sodium hexanitrite; ammonium sulphide as entering reagent.

Fig. 8. Test tube containing 5 per cent. "Difco" gelatin, cobalt reagent 1: 20; entering reagent, ammonium sulphide 1: 5. Gas bubbles, followed by colloidal fracture.

Fig. 9. Similar experiment but with cobalt reagent 1: 5. Distortion of bubbles according to gradient of swelling.

Fig. 10. One half of a capillary tube in which the bubbles form a simulacrum of periodicity. They show distortion due to swelling of the column of gelatin, in amount according to position. The middle point of the tube is above. A blue ring occurs at one end of the 5th visible bubble from the mouth of the tube. Sheet gelatin 5 per cent.—cobalt reagent 1: 16; entering reagent, ammonium sulphide (old) 1: 5. For the form of these bubbles see fig. 12.

Fig. 11. A single bubble in a capillary tube enlarged by spasmodic fracture, simulating periodicity.

Figs. 12, 13. Gelatin 10 per cent., cobalt reagent 1: 32 in tube, ammonium sulphide entering, 1: 10.

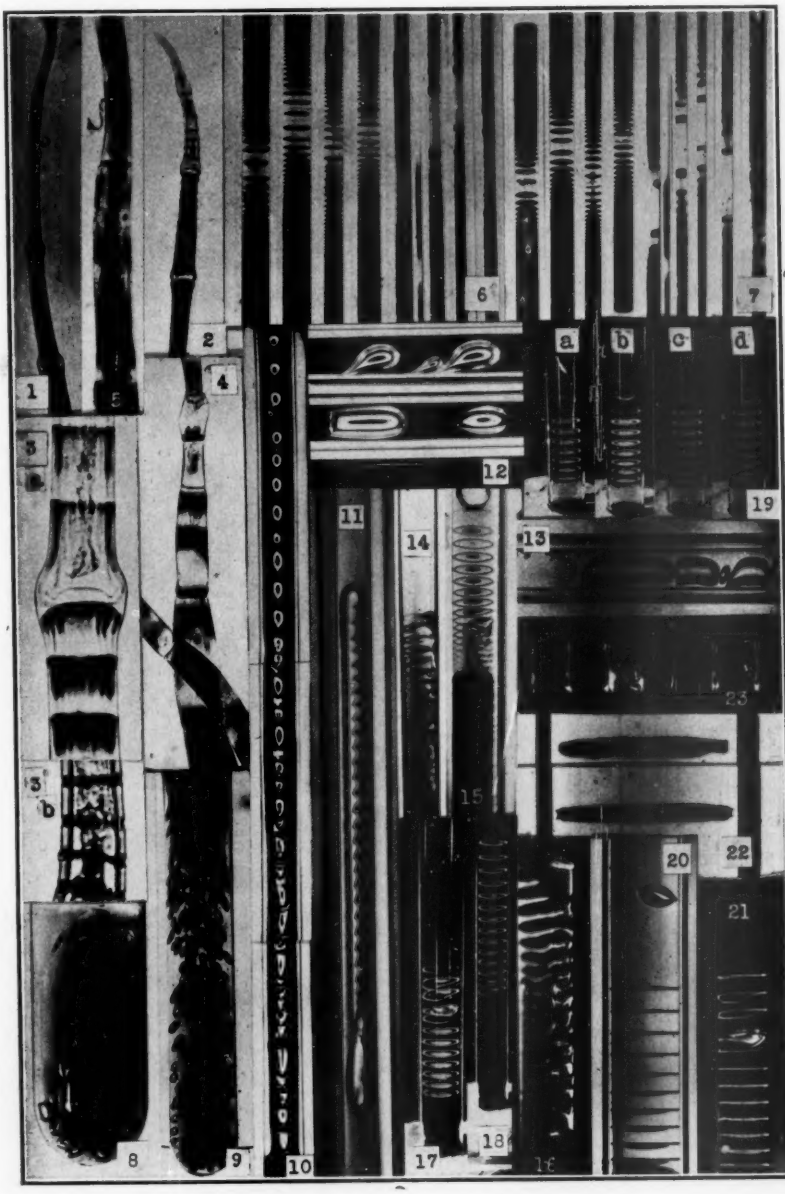
Fig. 12. Bubbles in capillary tubes seen from two points of view. They appear to have originated at the glass wall. With enlargement, displacement due to swelling of the gelatin column.

Fig. 13. Capillary tube with a cobalt ring-disc fractured by strain of gelatin or by passage of a bubble of gas.

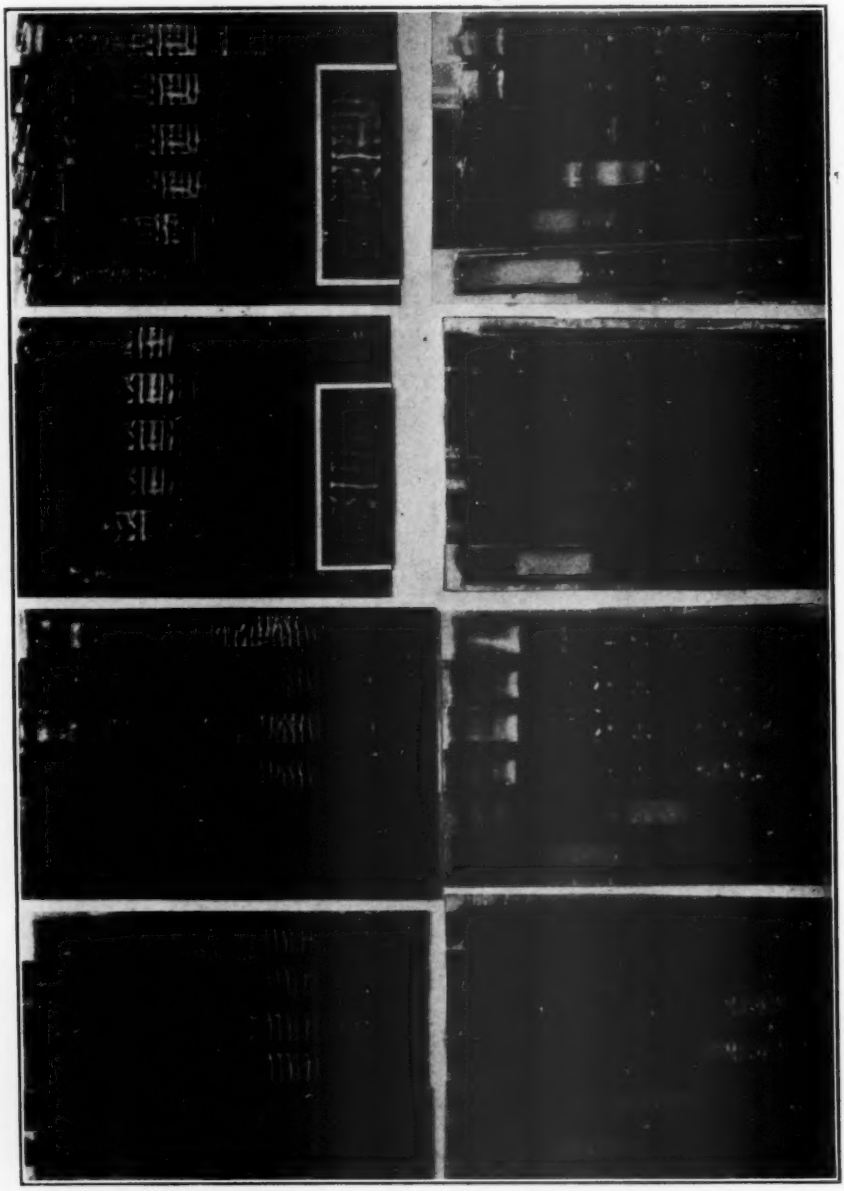
Figs. 14–23 incl., 32–34 incl. Tubes 2 mm. diam., 10 per cent. "Difco" gelatin; 5 per cent. freshly prepared cobalt reagent; entering reagent, ammonium sulphide 1: 10. Blue cobaltous hydroxide rings followed by black cobalt sulphide rings.

Figs. 14–15. Periodic precipitations of cobaltous hydroxide in advance of cobalt sulphide. Ring-and-disc structure traceable before periodicity becomes evident.

Fig. 16. Photograph somewhat later of the tube as seen in fig. 14, but by reflected light, bringing out the surface pattern more plainly.



LLOYD AND MORAVEK—PERIODIC PRECIPITATION



LLOYD AND MORAVEK—PERIODIC PRECIPITATION

FIGS. 17–18. Successive photographs of the same preparation by reflected light, viewed obliquely. Reduction and enlargement of discs, and distortion due to bubble.

FIG. 19. Four successive photographs to show (a) the first appearance of a ring, (b) followed by its disc, (c) their increase in density or thickness, and (d) the appearance of the next following ring.

FIG. 20. Displacement of discs due to swelling caused by entering reagent. Transmitted light.

FIG. 21. The same tube somewhat later. Distortion of successive rings in an oblique sense caused by the bubble seen above in fig. 20. Reflected light.

FIG. 22. Cobaltous hydroxide ring just after being completed (above), and after contracting (below).

FIG. 23. Blue cobaltous hydroxide rings covered by a *white* precipitate. Photographed through a blue absorbing filter.

FIGS. 24–31. Eight tubes containing 20 per cent. "Difco" gelatin, 5 per cent. cobalt reagent. The tubes of each sextette are regarded as numbered from 1 to 6 from the bottom, in each case. Tubes nos. 1, after 25 hours; nos. 2, after 52 hours; nos. 3, after 100 hours; nos. 4, after 114 hours; nos. 5, after 148 hours; nos. 6, after 244 hours.

FIGS. 24–25. Entering reagent ammonia 1: 20.

FIGS. 26–27. Entering reagent, ammonium sulphide 1: 20.

FIGS. 28–29. Entering reagent, ammonia 1: 5.

FIGS. 30–31. Entering reagent, ammonium sulphide 1: 5.

FIGS. 24, 26, 28, 30. At room temperature throughout period of observation.

FIGS. 25, 27, 29, 31. In cold (3° C.) (tubes 1–3), afterwards (tubes 4–6) at room temperature.

FIGS. 24, 26. Insets, enlargements of precipitate in tubes nos. 3 of the sextettes of these figures.

FIGS. 32–34. Showing secondary changes which take place in the cobalt sulphide rings. The blue rings (seen as white bands in advance of the black column) are persistent and become blackened. Compare with figs. 64, 69, which are these rings set free.

FIG. 35. Test tube with very crowded sulphide rings. *Cf.* fig. 38, which is a thin longitudinal section after removal from tube by breaking away the glass. 10 per cent. "Difco" gelatin; cobalt reagent 1: 10. Entering reagent ammonium sulphide 1: 10.

FIG. 36. Cobalt sulphide rings in a capillary tube, pushed away from the mouth of the tube. Before the swelling of the gelatin column they were indistinguishable, and are yet, where swelling has not intervened.

FIG. 37. Rings (true) and plates of cobalt hydroxide in advance of cobalt sulphide. The plates are formed by filling up the space by centripetal spreading of the ring in capillary tubes.

FIG. 38. See under fig. 35.

FIG. 39. In advance of black rings-discs is a brown zone free of precipitate and free from blue rings which have disappeared by dissolution. Entering reagent at first ammonia, for which, later, after the blue system was complete, ammonium sulphide was substituted.

FIG. 40. Test tube photographed from three positions to avoid parallax as far as possible. Cobaltous hydroxide.

FIG. 41. A series of tubes of various sizes containing a gelatin-ammonium sulphide mixture. Entering reagent, air. Lowermost tube, control, its upper end (on the right) hermetically sealed.

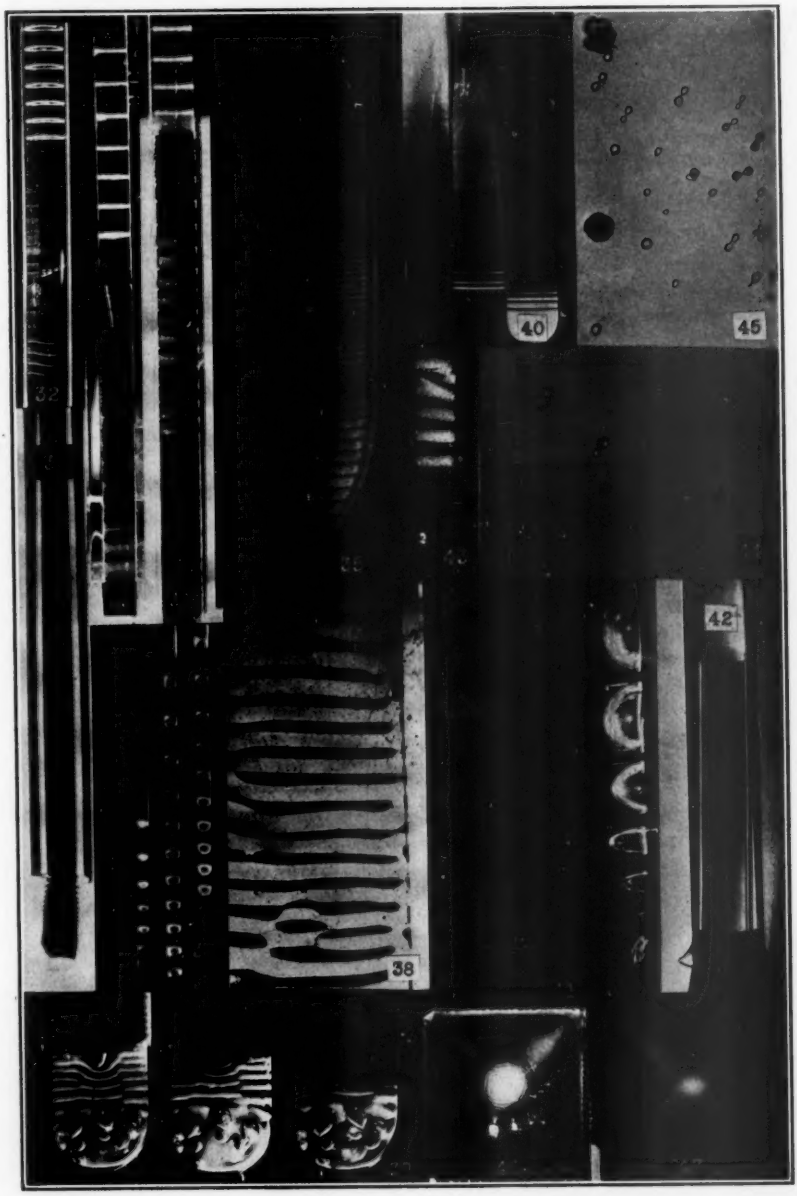
FIGS. 42–43. Tube filled with cobalt reagent 0.5 per cent., gelatin 20 per cent. mixture. Entering reagent: ammonia diluted 1: 50 (fig. 42), followed by ammonia diluted 1: 10, after which microcrystalline sulphur precipitated out in rings (fig. 43).

FIG. 44. Dumb-bell shaped microcrystalline sulphur obtained from rings of the tubes shown in fig. 41.

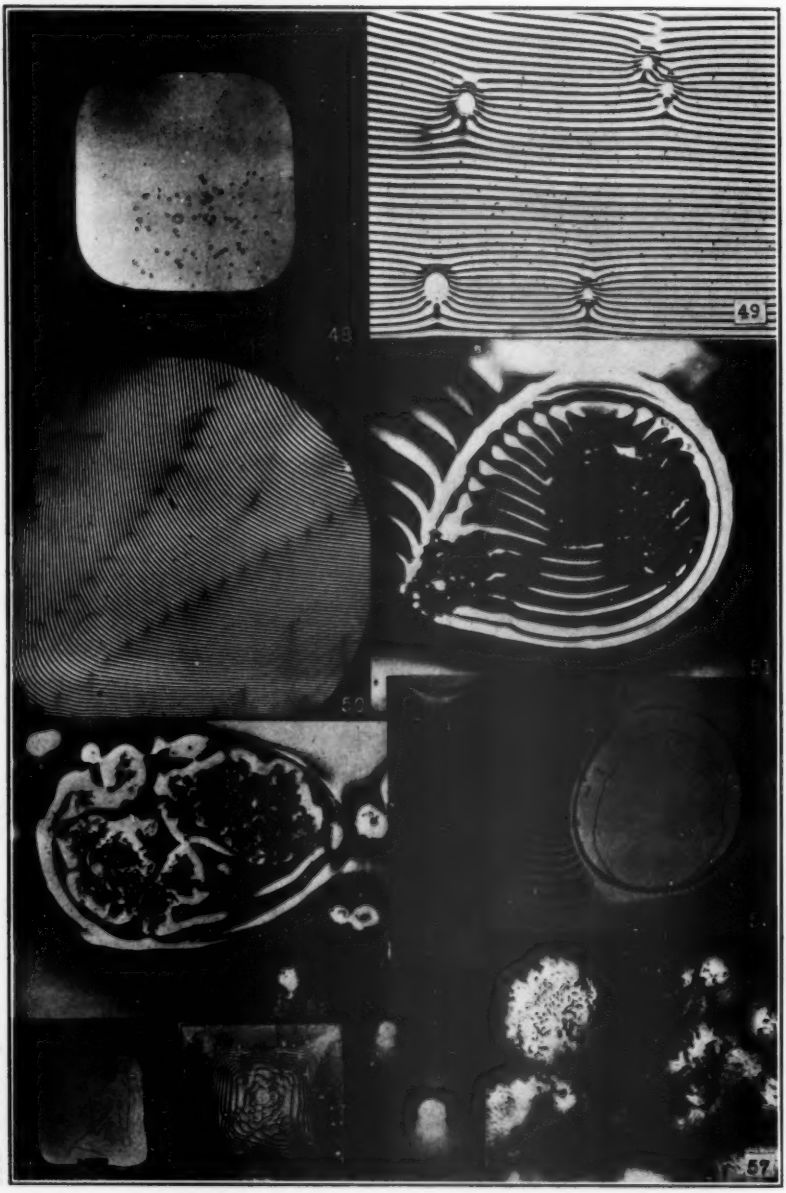
FIG. 45. Microcrystalline sulphur from the tube shown in fig. 43.

FIG. 46. Diffusion accompanied by periodic precipitation between glass plates inclosing gelatin 10 per cent. ("Difco") + 5 per cent. cobalt reagent. Entering agent, ammonium sulphide 1: 50.

FIG. 47. "Cross" diffusion pattern between glass plates, without visible periodicity. Between plates: "Gold label" gelatin 10 per cent. + cobalt 1: 32; entering reagent, ammonium sulphide 1: 10.



LLOYD AND MORAVEK—PERIODIC PRECIPITATION



LLOYD AND MORAVEK—PERIODIC PRECIPITATION

FIG. 48. Between glass plates: gelatin 10 per cent. ("Difco") + cobalt reagent 1: 10; entering reagent ammonium sulphide 1: 10 + ammonia.

FIG. 49. Between glass plates: gelatin 10 per cent. ("Difco") + cobalt 1: 10 + acetic acid; entering reagent, ammonium sulphide 1: 10.

FIG. 50. Detail from corner of fig. 48.

FIGS. 51, 52. Between glass plates: gelatin 10 per cent. ("Difco") + cobalt reagent 1: 10 with gypsum mixed in; entering reagent, ammonium sulphide 1: 5.

FIG. 53. Between glass plates: gelatin 5 per cent. ("Difco") + cobalt reagent 1: 5; entering reagent, ammonium sulphide, 1: 5.

FIG. 54. Between glass plates: gelatin 10 per cent. ("Difco") + cobalt reagent 5 per cent.; allowed to set 24 hours at 13° C. Entering reagent freshly prepared 1: 10. Cobaltous hydroxide rings visible beyond advancing margin of cobalt sulphide. The same preparation photographed later is seen in the next figure.

FIG. 55. Cobalt sulphide rings now accentuating the pattern formed by the cobaltous hydroxide rings seen in fig. 54.

FIG. 56. Set up as in fig. 53, but a thicker layer of the gelatin.

FIG. 57. Cobalt sulphide rings deposited at a gelatin-oil interface secondarily changed by evaporation of the metastable precipitate accompanied by the growth of minute, feathery sulphur crystals. Set up as for fig. 51, but oil droplets instead of gypsum.

FIG. 58. Tube = 6 mm. diameter containing gelatin 20 per cent. ("Difco"), cobalt reagent 1: 20 with a glass rod running approximately along the axis. Entering reagent, ammonia 1: 5.

FIG. 59. The same, but entering reagent, ammonium sulphide 1: 10.

FIG. 60. Glass rod after removal from the tube (fig. 59), on the left. The rod from tube, fig. 58, on the right.

FIG. 61. Portion of fig. 60 (left hand figure), enlarged.

FIG. 62. Same as fig. 58, but glass fragments instead of a rod.

FIG. 63. Detail from fig. 62 highly magnified. Precipitate is seen attached to glass fragments.

FIG. 64. Rings and discs of both cobaltous hydroxide blackened with cobalt sulphide (small ones), and cobalt sulphide rings and discs (larger ones); the shorter arrow points to the latter.

FIG. 65. Capillary tubes showing rings (strictly understood) with increasing width. Displacement of one ring due to a bubble.

FIGS. 66–68. Longitudinal sections of the gelatin column from test tubes containing cobaltous hydroxide precipitate, showing ring-and-disc structure.

FIG. 69. Portion of gelatin column from capillary tube (fig. 32) after being expelled therefrom by swelling. On the right: separated rings and part of a disc.

FIG. 70. Transverse section of gelatin column of test tube, fig. 40, near bottom of tube where the rings are separate, showing ring-and-disc structure. The disc is not completely circular, and has a pore in it, besides being of uneven thickness. The incompleteness of the ring is due to difficulty in complete removal from the glass wall.

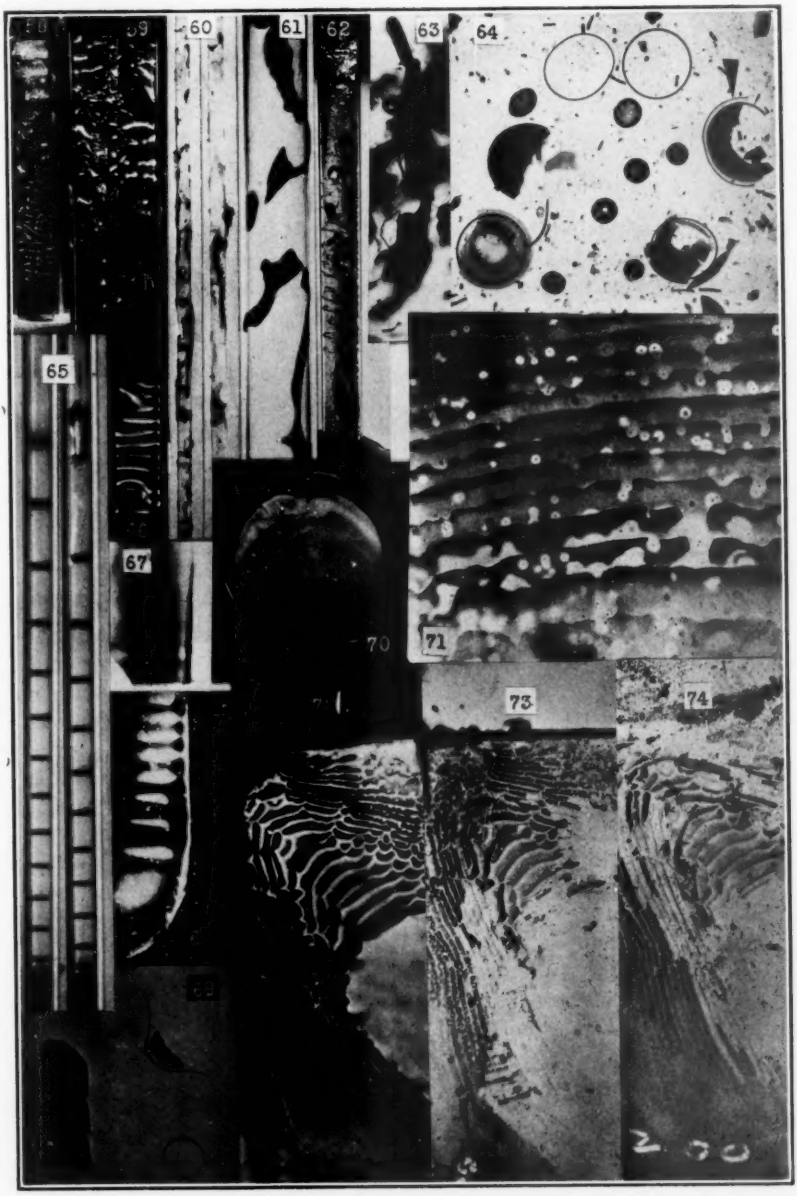
FIGS. 71–74. Periodic precipitation between glass plates from fluid cobalt reagent alone (without gelatin), with equal parts of ammonium sulphide and glycerine as entering reagent.

FIG. 71. Similar deposition forms of periodic precipitation between glass plates slightly displaced. The alternate rings in sharp focus are on the upper glass face; those out of focus, on the lower.

FIG. 72. Another precipitation, viewing the whole precipitate.

FIG. 73. That on the upper glass plate of fig. 72.

FIG. 74. That on the lower glass plate of fig. 72.



LLOYD AND MORAVEK—PERIODIC PRECIPITATION

CHANGES IN THE BUFFER SYSTEM OF THE WHEAT PLANT DURING ITS DEVELOPMENT

ANNIE M. HURD-KARRER

(WITH TWELVE FIGURES)

Introduction

In an earlier paper (**5**) it was reported that progressive changes occur in the acidity of the wheat plant during its seedling and maturation stages. Subsequent studies involving the complete titration curve have given a more comprehensive idea of the changes which take place in the titratable compounds of the juice. The results show a remarkably ordered relation between the buffer capacity of the juice, its hydrogen-ion and "titratable-acid" concentration, and the stage of development of the plants.

Methods

The varieties, White Odessa, a winter common wheat, and Jenkin, a spring club wheat, were grown for this investigation in a greenhouse at the Arlington Experiment Farm. At predetermined intervals, some of the plants were cut and the juice expressed and titrated electrometrically as rapidly as possible, according to a uniform procedure. Ten cubic centimeters of the undiluted juice were taken for each titration. As soon as the hydrogen electrode had come to equilibrium with the juice, *i.e.*, after about 20 minutes, the sample was titrated with N/20 sodium hydroxide. Immediately following the alkali titration a second 10-cc. sample of the juice was similarly titrated with N/20 sulphuric acid. The electrode was replatinized for each titration. Details of procedure are given in an earlier paper (**4**).

The potential differences measured after the addition of each successive cubic centimeter of alkali or of acid to the juice were translated into pH values and plotted against the quantities of alkali or acid producing them. The "blank" corrections (**11**) for these values, *i.e.*, the quantities of alkali required to bring equivalent volumes of water alone to the same pH values, were so small over the range of these titrations that they could be ignored. Only actual observations are plotted in the graphs.

Results

1. CHANGES IN THE FORM OF THE TITRATION CURVE DURING THE GROWTH PERIOD

For the experiments of 1926 the seed was sown in January, part of it being broadcasted for the work on very young seedlings, and the rest spaced

in rows. The surface soil was removed to a depth of about four inches and replaced by soil carefully sifted and mixed to insure uniformity. The temperature[1] and soil moisture were kept as favorable for the growth of wheat as possible. It was necessary to maintain fairly uniform moisture conditions in order to obtain comparable titrations.

Material for the measurements was collected about every other day for the first two weeks after the emergence of the seedlings, and weekly or

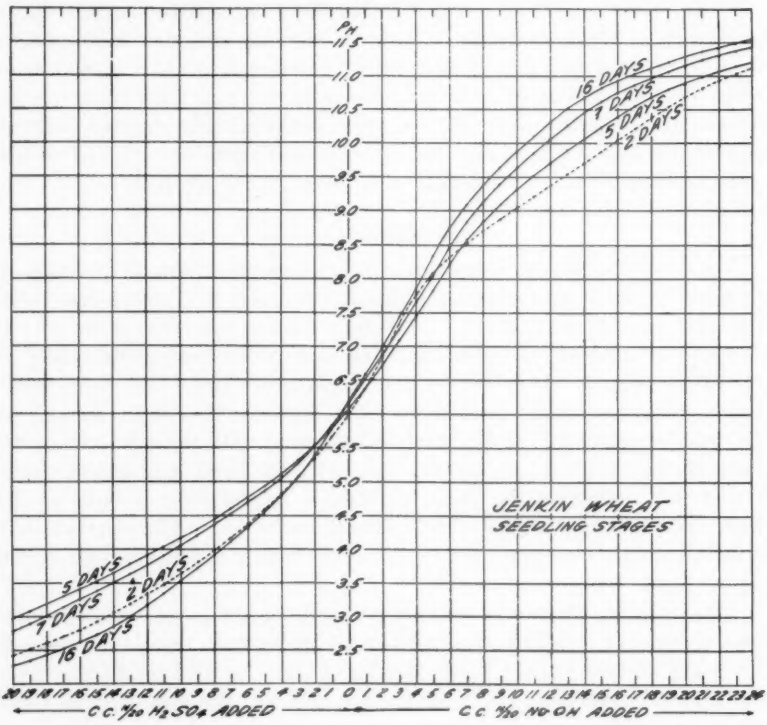


FIG. 1. Titration curves showing changes in the composition of juice of wheat (Jenkin) during the seedling stage.

[1] The air temperature was kept close to 16° C. during January, February, and March, falling to about 13° at night, and occasionally rising above 20° on warm afternoons. The soil temperature remained near 15° during this period. In April and May the air temperature was higher, frequently rising to 35° in the afternoon, and falling to 16° at night. The soil temperature fluctuated daily between 16° and 21°. During June, while the plants were maturing, the air temperature was frequently above 40°, with the soil temperature ranging from about 18° on the coolest days to 26° on the warmest days.

biweekly thereafter until the plants matured. The plants were cut just above the ground, the dead or dying lower leaves and culms of old plants being discarded. The heads were removed from plants in the booting stage and later. Age was reckoned from the date of emergence.

In figs. 1 and 2 are plotted the titration values obtained during the seedling stage. The curves show that a progressive change in the buffer

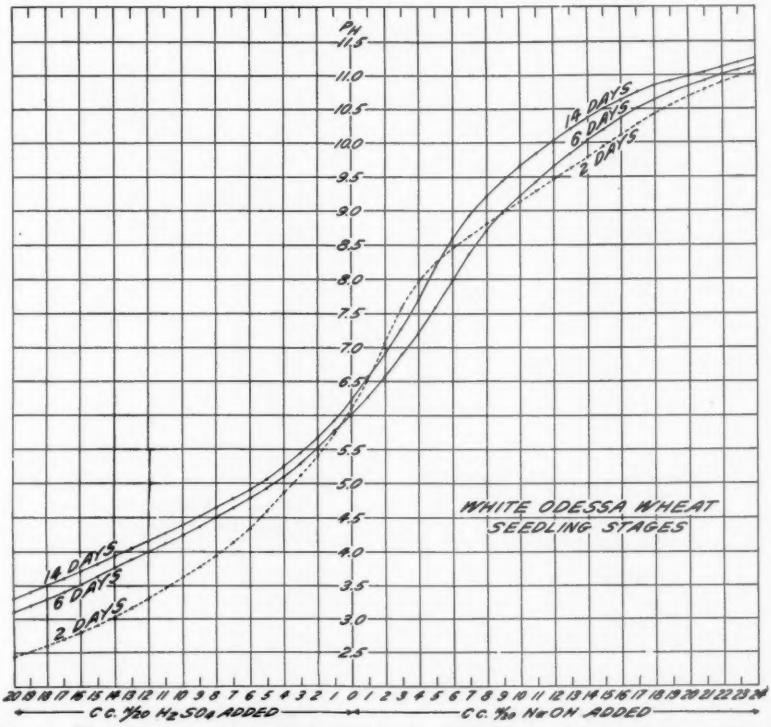


FIG. 2. Titration curves showing changes in the composition of juice of wheat (White Odessa) during the seedling stage.

system occurs during the first two weeks after emergence. When the plants are not over one inch high, with the first leaf not yet unfolded, the curve is very much flattened between about pH 8.5 and 10.5.[2] In fact, at a still earlier stage, when the plants are just showing above the ground, a curve may be obtained which is slightly depressed between these limits, as shown

[2] Broken lines are used in drawing the curves for these young seedlings wherever it is desirable to differentiate them more clearly from the curves for the older seedlings.

in fig. 3. Then in a few days this section of the curve becomes distinctly convex. The point of inflection changes from about pH 7.5 to a point near 8.0. The degree of convexity increases regularly through the 1-leaf and 2-leaf stages, or until about two weeks after emergence. Over the acid range, the curves become less and less steep as the plants develop. Thus during the seedling stage the buffer capacity of the juice is increasing in the

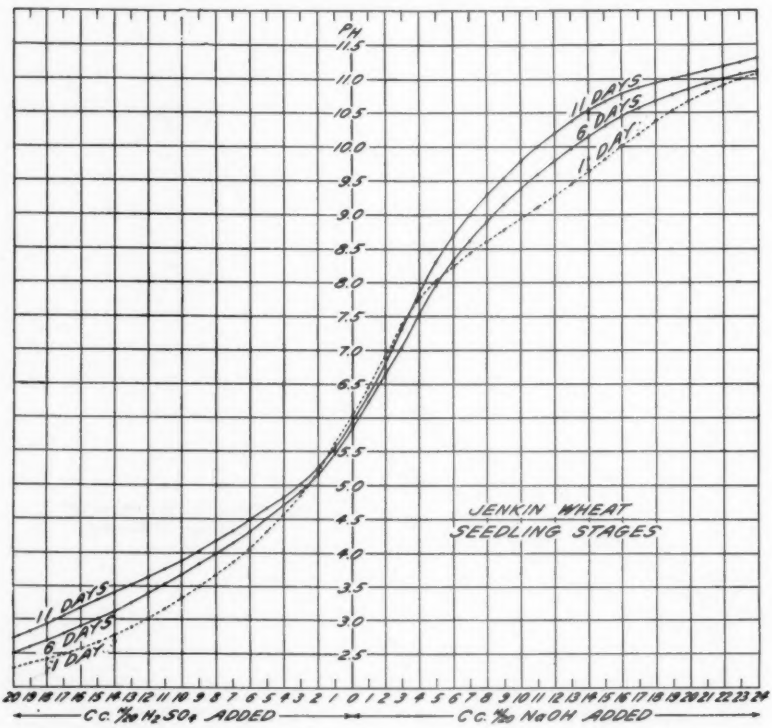


FIG. 3. Titration curves showing changes in the composition of juice of wheat (Jenkin) during the seedling stage.

acid range of the titrations while it is decreasing in the alkaline range.

The sequence of changes shown in figs. 1 and 2 is essentially the same in seedlings grown under quite different environmental conditions. In figs. 3 and 4 are shown corresponding sets of curves obtained in a preliminary experiment in 1925, with plants grown from seed sown three months later in the season (April) in a different greenhouse.

It appears from these curves that during the period of nutritional adjustment, between the emergent and the late-seedling stages, there occurs a change in the chemical composition of the juice which is reflected in the changing form of the titration curve. Curves for etiolated seedlings were found to resemble those for the emergent seedlings, being similarly flattened, and usually distinctly depressed, between pH 8.5 and 10.5. It is concluded, therefore, that assimilatory processes are not yet fully established in the

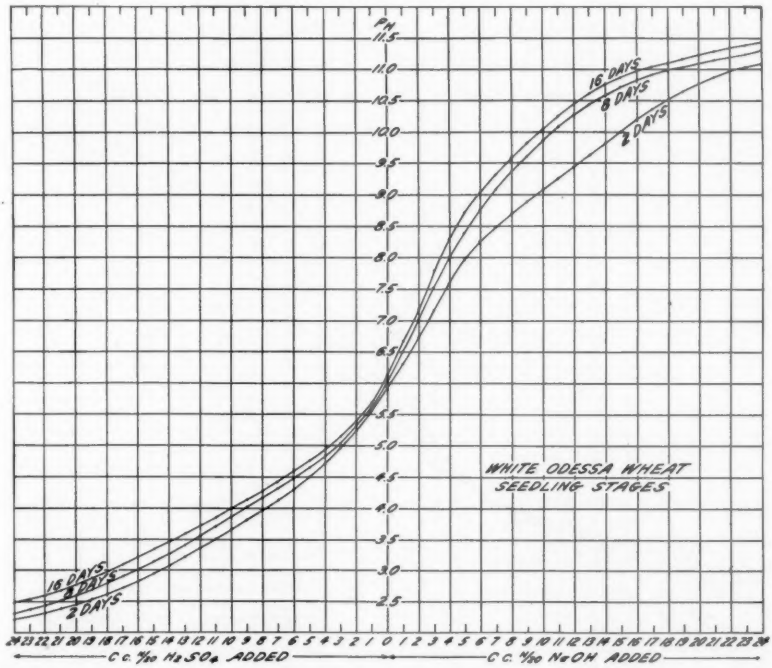


FIG. 4. Titration curves showing changes in the composition of juice of wheat (White Odessa) during the seedling stage.

very young greenhouse seedlings, even after they become green. The subsequent curves, increasingly convex between pH 8.5 and 10.5, evidently show the transition from the stage of complete dependence on the endosperm, to the stage of full photosynthetic activity. The curve of maximum convexity then indicates the stage when the constituents of the juice have reached an equilibrium concentration. This curve may be considered as marking the end of the seedling stage from a physiological standpoint.

Throughout the tillering stage and during the greater part of the shooting stage there are only small fluctuations in the position of the curve, probably caused by unavoidable variations in environmental conditions. The curves for this period lie so close to those of the late seedling stage that they can not be easily distinguished when plotted on the same chart. Therefore they are not included in the figures.

The first significant departure from this type of curve was obtained toward the end of the shooting stage. The juice became more highly buf-

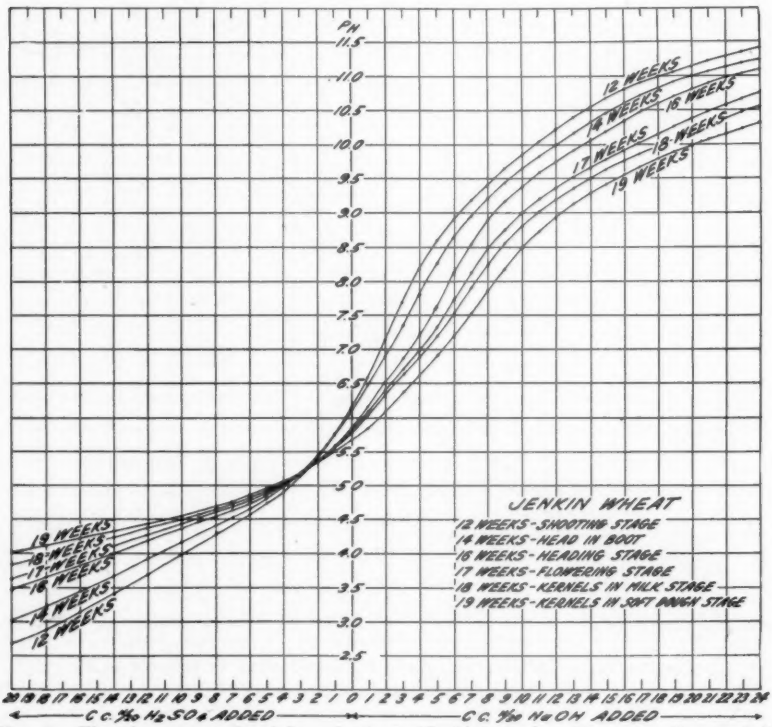


FIG. 5. Titration curves for juice of wheat (Jenkin) during the maturation period.

fered against both alkali and acid. This change marks the beginning of a period which continues throughout the stages of heading, flowering, and kernel development, during which there is a progressive increase in the buffer action of the juice (figs. 5 and 6[3]).

[3] Curves obtained for plants of White Odessa 13 to 16 weeks old were omitted from fig. 6 because they lay too close to each other, and to those obtained at 12 and 17 weeks, to be distinguishable. A curve representative of plants 20 weeks old also was omitted, as it was practically the same as the curve for 19 weeks.

The successive changes in the titration curve of White Odessa were less regular in the alkaline range than were those of Jenkin, although the same correlation of age with degree of buffer action was evident. White Odessa grew less normally and matured more slowly under greenhouse conditions than Jenkin, requiring 22 weeks to reach the soft-dough stage of the kernels as compared to the 19 weeks required by Jenkin to reach the same stage.

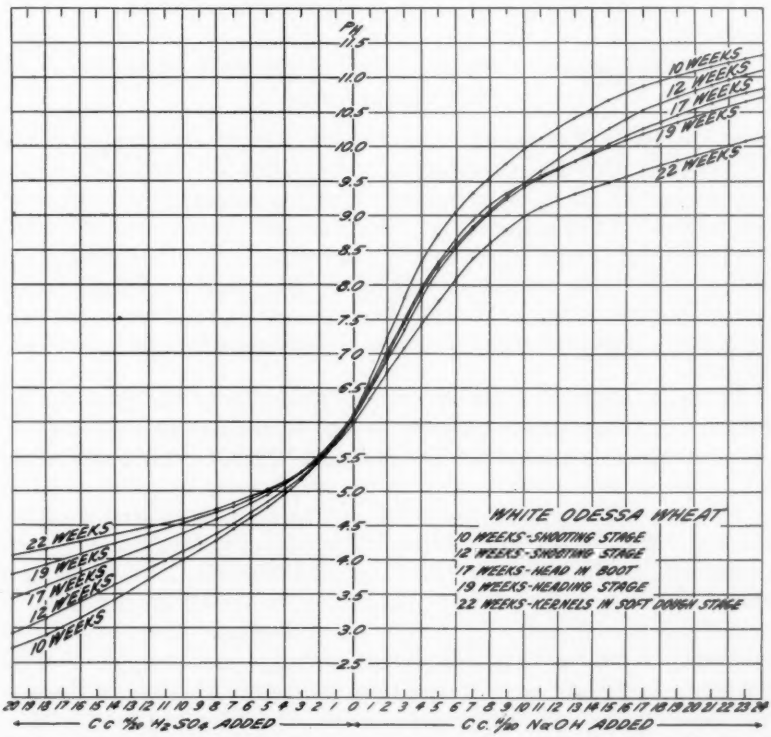


FIG. 6. Titration curves for juice of wheat (White Odessa) during the maturation period.

Also, White Odessa became slightly infected with mildew, which may have contributed to the greater irregularity of the change in the curve.

Figures 5 and 6 show that the curves for both Jenkin and White Odessa intersect between pH 5.3 and 6.0; that each curve passes through a point of inflection near pH 8.0; and that the slopes of the curves over both the acid and alkaline ranges decrease with increasing age of the plants.

The interrelationships of these curves suggest an analogy with the intersecting curves obtained on titrating different concentrations of a simple buffer solution such as the phthalate, phosphate, or standard acetate mixture. In the region of their intersection the curves resemble also those of SØRENSEN (**10**) which represent titrations of different concentrations of egg albumin. Thus it appears that the differences in buffer capacity shown from week to week during this period were due to progressive changes in the concentration of the juice as the plants ripened and dried.

2. THE CHANGE IN CONCENTRATION OF THE JUICE DURING THE MATURATION PERIOD

It is a matter of common observation that during the maturation period the tissues of the wheat plant become less and less succulent until, by the time the kernels are in the soft-dough stage, comparatively little juice can be expressed. The changes in the "titratable acid" values observed during this period were found (**5**) to be correlated with the rate of drying of the tissues. These observations as well as the characteristics of the curves themselves made it appear probable that the changes in buffer action were the result of increasing concentration of the juice and not due to metabolic changes.

In order to determine whether the concentration of the juice changes during this period, series of specific gravity measurements were made between the shooting and the soft-dough stages. Plants of both Jenkin and White Odessa were grown in 1927 in the same greenhouse, and under as nearly as possible the same conditions, as those maintained for the experiments of 1926. At intervals during the maturation period, juice was expressed and titrated, and its specific gravity determined by means of a small pycnometer. The juice was clarified for the specific gravity measurements by heating it carefully to 60° C. and filtering. The possibility of appreciable error resulting from the precipitation of solutes by this temperature was thought more remote than the danger of changing the concentration by filtering through paper pulp. The data are given in tables I and II and in fig. 7.

These data show that the "titratable acid" in the juice increases during the maturation period, and that the specific gravity increases, in general, at a corresponding rate. That the rate of increase in "titratable acid" is determined largely by the increase in density is shown by the fact that the ratio of the approximate weight of solids in a given volume of juice (the specific gravity measurement minus one) to the titratable-acid measurement is so nearly the same at each stage of development. It is interesting to note

that the buffer system functioned so effectively during this period that the hydrogen-ion concentration remained fairly constant up to the final measurement.

TABLE I

THE pH, "TITRATABLE ACID," AND SPECIFIC GRAVITY VALUES FOR JUICE OF JENKIN WHEAT DURING THE MATURATION PERIOD (EXPERIMENTS OF 1927)

AGE IN WEEKS	STAGE OF DEVELOPMENT	pH	"TITRATABLE ACID," (CC. N/20 NaOH REQUIRED TO REACH pH 8.3) A	SPECIFIC GRAVITY B	$\frac{B-1}{A}$
13	shooting	5.77	6.2	1.0269	0.0043
14	shooting	5.77	6.4	1.0219	0.0034
15	heads in boot	5.74	7.0	1.0334	0.0048
15½	heading	5.78	7.2	1.0318	0.0044
16	heading	5.74	7.4	1.0312	0.0042
17	flowering	5.77	10.4	1.0461	0.0044
18	kernels in milk stage	5.76	11.9	1.0576	0.0048
19	kernels in soft-dough stage	5.71	10.0	1.0491	0.0049
20	kernels in soft-dough stage	5.66	11.6	1.0557	0.0048

Specific gravity measurements thus show that the change in form of the titration curve during the maturation period is correlated with increasing concentration of the juice. Consequently the first departure from the type

TABLE II

THE pH, "TITRATABLE ACID," AND SPECIFIC GRAVITY VALUES FOR JUICE OF WHITE ODESSA WHEAT DURING THE MATURATION PERIOD (EXPERIMENTS OF 1927)

AGE IN WEEKS	STAGE OF DEVELOPMENT	pH	"TITRATABLE ACID," (CC. N/20 NaOH REQUIRED TO REACH pH 8.3) A	SPECIFIC GRAVITY B	$\frac{B-1}{A}$
12	shooting	5.74	7.8	1.0249	0.0032
13	shooting	5.81	7.9	1.0260	0.0033
15	shooting	5.74	8.0	1.0287	0.0036
16	shooting	5.81	8.4	1.0336	0.0040
17	heading	5.73	11.0	1.0455	0.0041
17	flowering	5.85	10.1	1.0470	0.0047
18	late flowering	5.77	13.2	1.0481	0.0036
19	kernels in milk stage	5.73	14.0	1.0566	0.0040
20	kernels in soft-dough stage	5.83	14.7	1.0690	0.0047
21	kernels in soft-dough stage	5.55	13.1	1.0594	0.0045

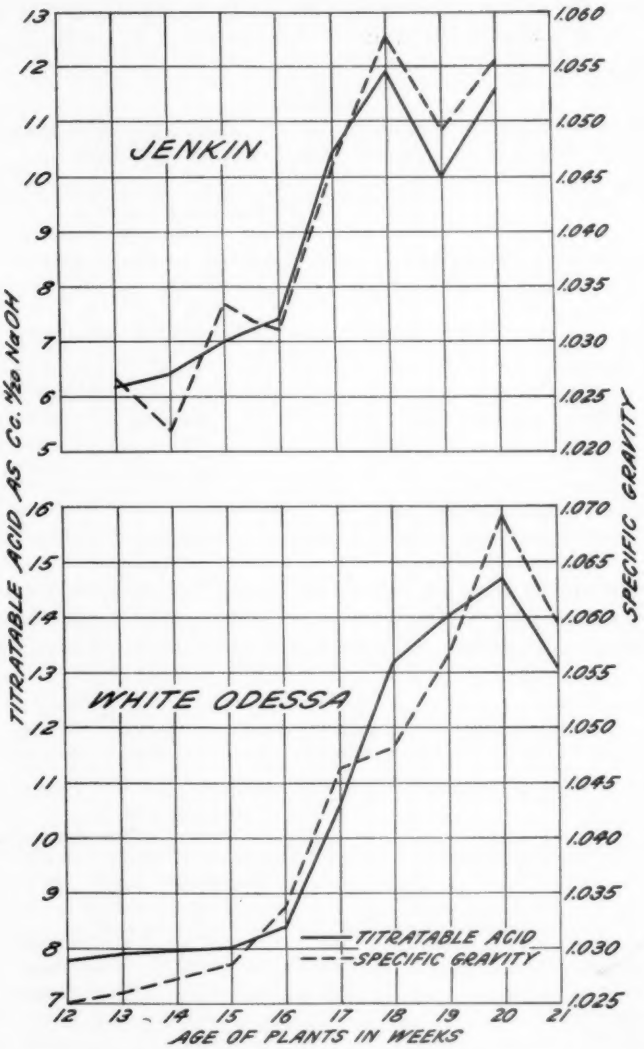


FIG. 7. Correlation between the titratable acid and specific gravity of juice of Jenkin and White Odessa wheat during the maturation period.

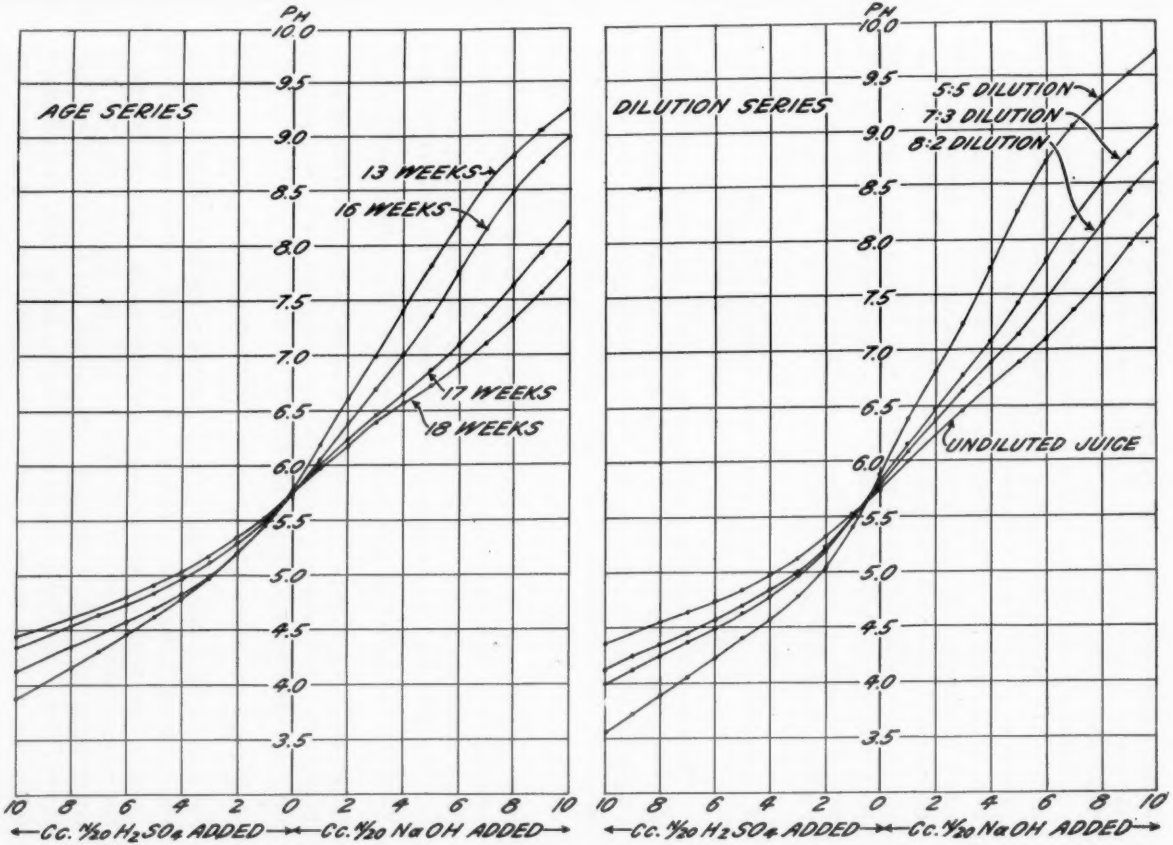


FIG. 8. Titration curves for juice of wheat (Jenkin) in different stages of development compared with artificially diluted juice.

of curve obtained at the end of the seedling stage, and also throughout the subsequent stages of tillering and shooting, marks the beginning of the maturation period.

If change in concentration is solely responsible for the differences in the curve at the successive stages of development, then artificial dilutions of juice from plants in a late stage of development should give curves similar to those obtained for the undiluted juice at earlier stages. Titrations of a series of dilutions of juice from 17-weeks-old plants is shown in fig. 8 together with four curves representing the titrations of undiluted juice from the plants at different stages of development. The resemblance between the two groups of curves is sufficiently close to indicate that change in concentration alone accounts for the change in the titration values observed during the maturation period.

3. Effects of the increasing concentration of the juice during the maturation period on its buffer values

The slopes of a titration curve show graphically the buffer capacity of a solution at each stage of titration. Over any given reaction range, the ratio of the pH increment, ΔpH, to the corresponding increment of alkali (or acid), ΔB, gives a measure of the buffer capacity. This ratio is more convenient than the ratio $\frac{dB}{dpH}$ suggested by Van Slyke (**12**), since ΔB is always equal to one, and it is just as useful for the purpose of the present investigation. It is defined as the change in pH produced by the addition of each cubic centimeter of N/20 NaOH or of N/20 H_2SO_4 to the juice during the titration. It is essentially the reciprocal of the buffer unit $\frac{\Delta(S - S_0)}{\Delta p}$ suggested by Koppel and Spiro (**6**), since the corrections for the effects of water alone (S_0) over the reaction ranges involved are practically zero. Inclusion of a correction for the dilution error is immaterial for values between pH 3.0 and pH 11.0 (**7**).

In fig. 9 are plotted the values of $\frac{\Delta pH,}{\Delta B}$ with the increments in alkali (or acid) as abscissae, for the series of titrations made in 1926 for Jenkin wheat during its maturation period. Since the buffer action at any given point in the titration varies inversely with the change in pH produced by the corresponding increment of alkali or acid, the highest point on each curve marks the point of minimum buffer action. It should be noted that the converse of this statement, namely, that the lowest point on each curve marks the point of maximum buffer action, is not true according to the usual meaning of the term buffer action, which refers to the action of the

solutes only. Over the regions of their flattened ends, where the reaction of the solution is changing most slowly, the titration curves approach asymptotically a line through the pH value of the reagent being added and parallel to the axis of abscissae. VAN SLYKE (**12**) refers to the slow rate of change of reaction in these regions as due to the buffer effect of water.

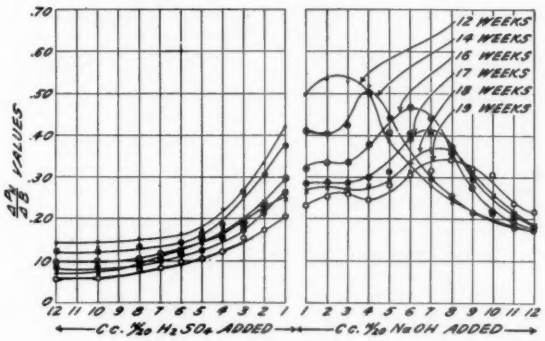


FIG. 9. Graphs showing change in $\frac{\Delta \text{pH}}{\Delta \text{B}}$ values for juice of wheat (Jenkin) during the maturation period.

Minimum buffer action occurs at a different point on each curve in fig. 9, but is found, by reference to the corresponding pH values (fig. 5), to be always near pH 8.0.[4] This constancy is evidence that no radical change takes place in the composition of the juice during the maturation period. The fact that the point of minimum buffer action occurs farther along the axis of abscissae in each successive titration is evidence of the increasing concentration of the juice as the plants ripen and dry.

Artificially diluting the juice of plants in late stages of development causes the buffer values to approximate those of undiluted juices of plants in earlier stages of development. In fig. 10-A are plotted the values of $\frac{\Delta \text{pH}}{\Delta \text{B}}$, with the increments of alkali (and acid) as abscissae, for the titrations shown in fig. 8 for diluted juice from nearly mature plants. In fig. 10-B are shown similar curves for the titrations of the undiluted juice of the plants when they were at different stages of development. The curves

[4] This point of minimum buffer action does not indicate exhaustion of the titratable materials in the juice, for the "blank" corrections show that it takes less than 0.1 cc. of the alkali to bring equivalent volumes of water alone to this reaction. In fact, the "blank" corrections are negligible over the entire range of these curves, showing that the titratable materials are far from exhausted at pH 8.0.

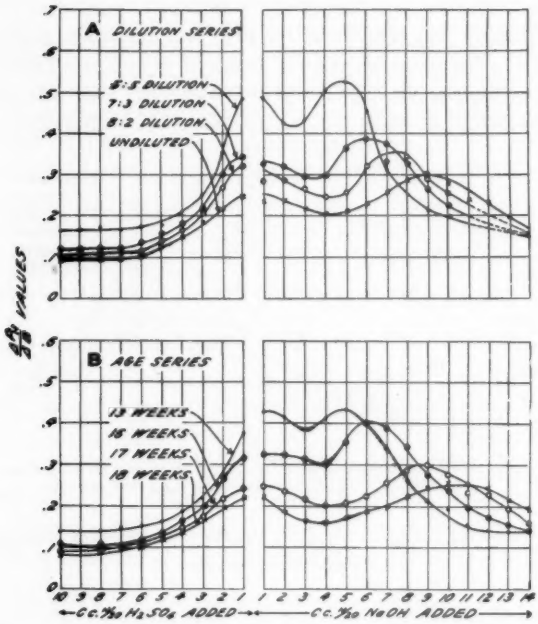


FIG. 10. $\frac{\Delta pH}{\Delta B}$ values for juice of wheat (Jenkin) in different stages of development and the corresponding values for a series of artificial dilutions of juice.

show that the buffer action varies with age in the natural juices as with concentration in artificially diluted juices.

Comparison of the undiluted juice of plants of different ages with the artificial dilutions is facilitated by the summary in table III. It is noteworthy that the hydrogen-ion concentration of the natural juice is practically the same (pH 5.7–5.8) at each stage of development, but is decreased appreciably by artificial dilution.

4. Preliminary studies on the nature of the titratable constituents in wheat juice

When added to a complex mixture like wheat juice, alkali and acid undoubtedly react with several types of compounds, all of which, if present in sufficient quantity, affect the course of the titration and the so-called "titratable acidity" values. These compounds include organic acids and bases with their salts, and organic and inorganic ampholytes. Little is known concerning the identity and the relative quantities of these compounds occurring in wheat juice.

The buffer systems investigated by HEMPEL (**3**), working on succulent plants, and YOUDEN and DENNY (**13**), on extracts of potato tubers, consist mainly of organic acids and their salts. LEUTHARDT (**7**) also has shown that the titratable compounds in fruits, including apples, grapes, lemons, and tomatoes, are largely organic acids and their salts. He found that sugars are responsible for considerable buffer action at pH values above 9.0.

TABLE III

COMPARISON OF WHEAT JUICE (JENKIN) FROM PLANTS OF DIFFERENT AGES BETWEEN THE SHOOTING AND SOFT-DOUGH STAGES WITH ARTIFICIAL DILUTIONS OF JUICE FROM PLANTS IN THE FLOWERING STAGE

AGE OF PLANTS IN WEEKS	SPECIFIC GRAVITY OR DILUTION	pH	"TITRATABLE" ACID (CC. N/20 NaOH REQUIRED TO REACH pH 8.3)	pH AT APPROXIMATE POINT OF MINIMUM BUFFER ACTION
A. Diluted juice	Dilution			
17	5 cc. juice + 5 cc. H_2O	5.90	5.1	8.0
17	7 cc. juice + 3 cc. H_2O	5.85	7.3	7.8
17	8 cc. juice + 2 cc. H_2O	5.81	8.4	7.8
17	10 cc. juice + 0 cc. H_2O	5.77	10.4	7.8
B. Natural juice	Sp. gr.			
13	1.0269	5.77	6.2	7.8
16	1.0312	5.74	7.4	7.8
17	1.0461	5.77	10.4	7.8
18	1.0576	5.76	11.9	7.8

Several investigators (**1, 2, 3, 7**) have called attention to the fact that the ampholytes in plant juices may act as buffers, but it is not known whether they are generally present in sufficient quantity to affect the titration appreciably. COHN, GROSS and JOHNSON (**2**) report that between pH 4.5 and 8.5 the form of the titration curve of the juice of potato tubers is largely determined by proteins, principally the globulin tuberin. YOUDEN and DENNY (**13**), however, have found that the proteins have but little effect on the titration of water extracts of this tissue. YOUDEN and DENNY (**13**) found also that a solution of glycocoll containing about the same amount of nitrogen as did the water extract of potato tubers showed comparatively little buffer action. LEUTHARDT (**7**), on the other hand, concluded that the characteristic form of the titration curve for the juice of *Mesembryanthemum* is due to glutamin, which is present in relatively large quantities.

Removal of the heat-coagulable proteins from wheat juice is easily accomplished by boiling and filtering. According to OSBORNE (**9**), there probably is very little protein left in an expressed juice after this treat-

ment. The slope of the titration curve for the boiled and filtered juice is slightly steeper than that of the raw, untreated juice (figs. 11, 12, table IV.)[5] The buffering capacity is reduced to practically the same extent in both the acid and alkali titrations. The effect is similar to that shown by

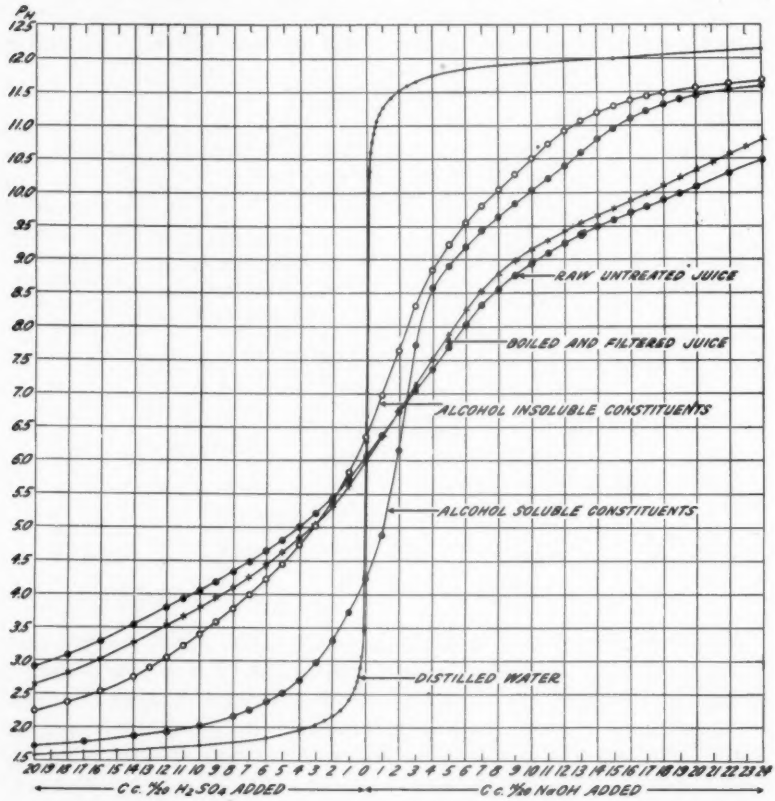


FIG. 11. Titration curves for Jenkin wheat (2 months old) before and after removal of the heat-coagulable and of the alcohol-soluble (hot 95 per cent.) buffers. The curves show that 95 per cent. alcohol dissolves some of the alkali-titratable material but very little of the acid-titratable material.

[5] The data from some of the experiments are presented in the form of graphs (figs. 11–12) and those from others are in tables (tables IV–V). In the case of an experiment such as that of table IV, not all the curves could be drawn in one figure without confusion. Thus the curves for the recombined fractions in the experiments of figs. 11 and 12 had to be omitted because they lay too close to the curve for the whole juice. It seemed desirable, therefore, to give the complete data in the form of tables for one or two typical experiments.

the corresponding curves of YOUDEN and DENNY (13) for an extract of potato tubers, except that these investigators apparently obtained a slightly greater reduction on the alkaline side of the curve than on the acid side. It is evident that the heat-coagulable substances play but a small part in the buffer system of the wheat plant.

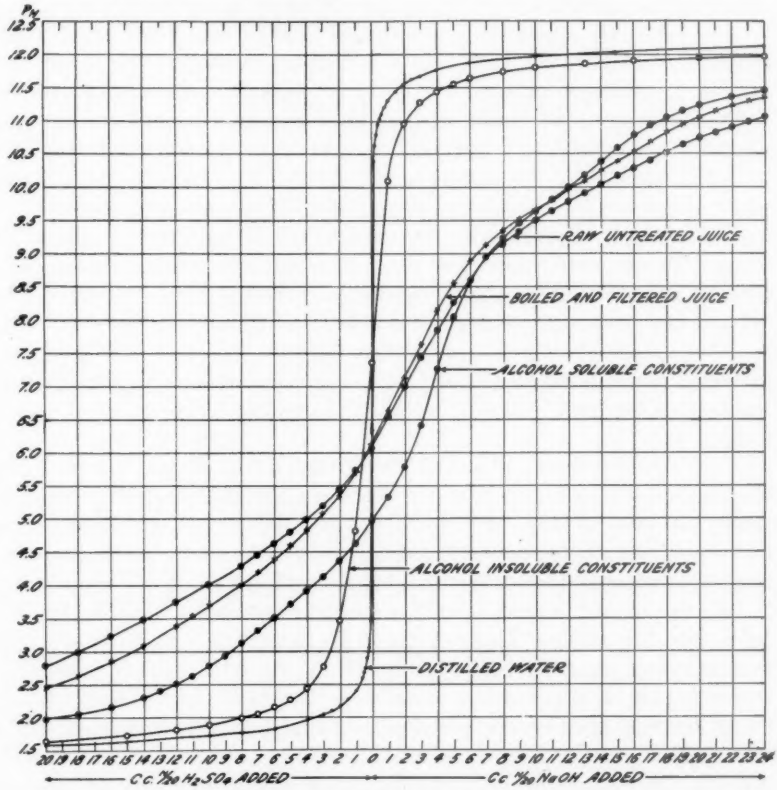


FIG. 12. Titration curves for White Odessa wheat (2 months old) before and after removal of the heat-coagulable and of the alcohol-soluble (cold 75 per cent.) buffers. The curves show that 75 per cent. alcohol dissolves much of both the alkali and acid-titratable material.

Since most of the free organic acids found in plants (8) are fairly soluble in alcohol, they may be largely removed from the juice by evaporating it almost to dryness and extracting with this solvent. However, the salts of these acids are not all soluble in alcohol, so that the alcohol-soluble

fractions could not show the full buffer capacity of the juice. The amino-acids which have been found in plants (8) are, with the exception of proline, but slightly soluble in 95 per cent. alcohol and practically insoluble in absolute alcohol. Polypeptides, also, are but slightly soluble in alcohol. These substances would be left for the most part in the alcohol-insoluble residue after extraction with 95 per cent. or absolute alcohol.

Alcoholic extractions of the evaporated juice of both Jenkin and White Odessa were made. In each case a measured volume of the boiled and filtered juice was evaporated almost to dryness on a steam bath and the residue thoroughly extracted with several portions of neutral redistilled alcohol. The extract was separated from the alcohol-insoluble material by filtration, the alcohol removed from the extract by evaporation on a steam bath, and the residue made up to the original volume with water. The alcohol-insoluble material was then redissolved in the original volume of water. Ten–cc. portions of these fractions were titrated with acid and with alkali. The fractions were then recombined, evaporated, made up to proper volume, and titrated for comparison with the original whole juice.

In one of the experiments reported in table IV, the evaporated juice was extracted successively with alcohol of different concentrations, the different fractions being kept separate, evaporated and made up to volume with water.

Cold absolute alcohol dissolved almost none of the titratable material of the juice, as shown by the fact that the values for this fraction were practically the same as those of distilled water. Cold 95-per cent. alcohol dissolved a little more of the buffer substances, but the curve for this fraction also was similar to that of distilled water (table IV).

Hot 95-per cent. alcohol extracted part of the material which buffers the juice over the alkaline range of the curve, but very little of that which buffers it over the acid range (tables IV, V, fig. 11). The hydrogen-ion concentration of this fraction was always very much higher than that of either the raw juice or the boiled juice, suggesting the presence of acids without their salts or without some of the buffers which are associated with them in the whole juice. The hydrogen-ion concentration of the alcohol-insoluble fraction was always much lower than that of the whole juice—lower even than that of distilled water. This fraction showed almost as great buffer action in the acid range of the curve as did the original boiled and filtered juice, and also considerable buffer capacity in the alkaline range, indicating that not all of the compounds involved in the buffer system are soluble in hot 95-per cent. alcohol.

Seventy-five-per cent. alcohol dissolved practically all of the buffers in the juice as shown by the fact that the shape of the curve for these fractions

TABLE IV

ELECTROMETRIC TITRATIONS OF JUICE FROM WHEAT PLANTS (WHITE ODESSA, 2 MONTHS OLD) BEFORE AND AFTER REMOVAL OF THE HEAT-COAGULABLE BUFFERS, AND OF THE ALCOHOL SOLUBLE AND ALCOHOL INSOLUBLE CONSTITUENTS

TABLE IV

ELECTROMETRIC TITRATIONS OF JUICE FROM WHEAT PLANTS (WHITE ODESSA, 2 MONTHS OLD) BEFORE AND AFTER REMOVAL OF THE HEAT-COAGULABLE BUFFERS, AND OF THE ALCOHOL SOLUBLE AND ALCOHOL INSOLUBLE CONSTITUENTS

CC. ALKALI AND ACID ADDED TO 10 CC. JUICE	RAW, UNTREATED JUICE	JUICE HEATED TO BOILING POINT AND FILTERED	RESIDUE, AFTER EVAPORATION OF HEATED AND FILTERED JUICE, EXTRACTED SUCCESSIVELY WITH COLD ALCOHOL OF DIFFERENT CONCENTRATIONS					RESIDUE EXTRACTED WITH COLD 75 PER CENT. ALCOHOL ONLY			RESIDUE EXTRACTED WITH HOT 95 PER CENT. ALCOHOL ONLY			DISTILLED WATER
			100 PER CENT. ALCOHOL SOLUBLE A	95 PER CENT. ALCOHOL SOLUBLE B	75 PER CENT. ALCOHOL SOLUBLE C	ALCOHOL INSOLUBLE D	RECOMBINED FRACTIONS A+B+C+D	ALCOHOL SOLUBLE A	ALCOHOL INSOLUBLE B	RECOMBINED FRACTIONS A+B	ALCOHOL SOLUBLE A	ALCOHOL INSOLUBLE B	RECOMBINED FRACTIONS A+B	
N/20 NaOH														
	pH	pH	pH	pH	pH	pH	pH	pH	pH	pH	pH	pH	pH	pH
0	6.09	6.14	4.46	4.19	4.99	7.64	5.04	4.97	7.32	5.30	3.97	6.43	5.64	6.30
1	6.53	6.66	10.80	9.93	5.35	10.87	5.34	5.33	10.08	5.64	4.76	7.11		11.30
2	6.99	7.12	11.32	10.99	5.79	11.32	5.72	5.79	10.94	6.07	6.57	7.80		11.55
3	7.43	7.62	11.54	11.36	6.43	11.51	6.22	6.40	11.26	6.63	8.38	8.54		11.69
4	7.85	8.13	11.65	11.53	7.40	11.63	6.92	7.25	11.43	7.31	9.12	9.08		11.78
5	8.26	8.55	11.73	11.66	8.19	11.72	7.71	8.04	11.52	8.03	9.56	9.50		
6	8.62	8.87			8.75		8.35	8.58	11.63	8.59	9.92	9.81		11.88
7	8.92	9.13	11.84	11.79	9.09	11.83	8.74	8.94		8.94	10.24	10.11		
8	9.14	9.33			9.35		9.00	9.22	11.74	9.20	10.55	10.37		
9	9.34	9.51			9.57		9.23	9.44		9.41	10.84	10.63		
10	9.50	9.66	11.94	11.89	9.76	11.90	9.40	9.63	11.80	9.60	11.06	10.87		11.98
11	9.64	9.80			9.95		9.55	9.80		9.78	11.22	11.06		
12	9.78	9.95			10.17		9.69	9.98		9.92	11.34	11.20		
13	9.90	10.09			10.39		9.87	10.17	11.88	10.09	11.44	11.31		
14	10.03	10.25			10.61		10.06	10.38		10.23	11.52	11.40		
15	10.16	10.39	12.01	11.98	10.81	11.95	10.22	10.59		10.37		11.49		12.04
16	10.27	10.54			10.96		10.43	10.77	11.92	10.52	11.62	11.54		
17	10.40	10.71			11.09		10.62	10.92		10.66				
18	10.52	10.82			11.18		10.78	11.04		10.78	11.69	11.63		
19	10.63	10.94			11.28		10.94	11.14		10.90				
20	10.74	11.05			11.36		11.06	11.23	11.95	11.00	11.74	11.69		
21	10.81	11.14					11.20			11.09				
22	10.89	11.22			11.47		11.28	11.36		11.17				
23	10.98	11.30					11.34			11.24				
24	11.05	11.35	12.08	12.03	11.56	12.01	11.41	11.45	11.97	11.30	11.81	11.78		12.13
N/20 H_2SO_4														
0	6.06	6.15	4.48	4.21	5.02	7.77	5.01	4.97	7.40	5.30	4.01	6.45	5.64	6.25
1	5.73	5.68	2.54	2.61	4.68	3.22	4.69	4.64	4.81		3.38	5.87		2.41
2	5.44	5.33	2.22	2.26	4.40	2.48	4.42	4.36	3.47	4.69	2.89	5.40	4.96	2.16
3	5.19	5.07	2.05	2.09	4.16	2.22	4.19	4.12	2.77		2.55	4.98		2.04
4	4.98	4.82	1.95	1.99	3.94	2.08	4.00	3.91	2.43	4.23	2.33	4.62	4.44	1.95
5	4.80	4.59	1.89	1.91	3.75	1.98	3.80	3.70	2.26	4.05	2.18	4.32		
6	4.62	4.38			3.54		3.63	3.50	2.14	3.87	2.07	4.05	4.03	1.83
7	4.45	4.19	1.79	1.81	3.35	1.85	3.48	3.31	2.05	3.69	1.99	3.80	3.87	
8	4.30	4.01			3.15		3.30	3.12	1.98	3.52	1.93	3.58	3.68	
9		3.85			2.97		3.14	2.93		3.35		3.35	3.51	
10	4.02	3.70	1.69	1.71	2.80	1.74	2.98	2.78	1.88	3.20	1.82	3.14	3.34	1.72
11		3.54			2.65		2.83	2.62		3.05		2.93	3.19	
12	3.75	3.40			2.51		2.69	2.50	1.81	2.90	1.76	2.74	3.04	
13					2.39		2.57	2.40		2.78			2.90	
14	3.49	3.09			2.30		2.47	2.30		2.68		2.47	2.78	
15			1.61	1.62		1.63	2.39		1.73	2.58				1.64
16	3.23	2.86			2.14		2.32	2.16		2.49	1.65	2.27	2.57	
17														
18	3.00	2.63			2.01		2.16	2.03		2.35		2.11	2.41	
19														
20	2.80	2.46	1.54	1.56	1.93	1.57	2.05	1.97	1.64	2.24	1.59	2.02	2.28	1.58

was very similar to that of the whole juice, and further, by the fact that the shape of the curve for the alcohol-insoluble residue remaining after extraction with this solvent closely approached that of distilled water (table IV, fig. 12). The results were practically the same whether the alcohol was hot or cold. It is interesting to note that the fractions containing the substances insoluble in 75-per cent. alcohol were alkaline, the hydrogen-ion concentration being not only lower than that of the original juice, but lower than that of distilled water.

One extraction experiment was carried out with some dried plants of the variety Purplestraw, of approximately the same age and condition as were the White Odessa and Jenkin plants. The plants were dried in an electric oven at 80° C., then ground to a powder in a hand mill. Thirty-five grams of the powder were extracted in 320 cc. distilled water by agitating the mixture for four hours on a shaking machine. The filtrate gave titration values very close to those for a fresh juice. A portion of the filtrate was then evaporated to dryness without previous boiling, and the residue extracted with hot 95-per cent. alcohol. After filtering, the alcohol was removed by evaporation on the steam bath and the residue redissolved in the proper volume of water. The titration values for the solution containing the alcohol-soluble constituents and for that containing the redissolved alcohol-insoluble residue are given in table V, together with the values for the recombined fractions and for the original whole extract. The results are similar to those obtained with fresh juice.

In all the experiments, the hydrogen-ion concentration of the recombined fractions was somewhat higher than that of the original whole juice (table IV) or extract (table V). This increase appeared to be due to changes which occurred during evaporation on the steam bath.[6] However, the buffer action of the extracts was not affected appreciably, as shown by the fact that the curve for the recombined fractions always approached very closely that of the original boiled and filtered juice. That the increase in hydrogen-ion concentration was not due to removal of alcohol-coagulable material was shown by the fact that a similar increase occurred in juice which was evaporated on the steam bath and redissolved in water without treatment with alcohol.

Summary

The electrometric titration curve of juice expressed from wheat plants changes progressively during the seedling stage and during the maturation period. Only minor changes, correlated with environmental factors, occur during the tillering stage and most of the shooting stage.

[6] A sample of juice which was evaporated by boiling at 64° in a partial vacuum and then made up to volume also became more acid. The original pH value of 5.82 was changed to 5.68.

TABLE V

ELECTROMETRIC TITRATIONS OF A WATER EXTRACT OF DRIED WHEAT PLANTS (PURPLESTRAW, 2 MONTHS OLD) AND OF ITS ALCOHOL (HOT 95 PER CENT.) SOLUBLE AND ALCOHOL INSOLUBLE CONSTITUENTS

ALKALI TITRATIONS						ACID TITRATIONS					
Cc. N/20 NaOH added to 10 cc. juice	Original extract	Alcohol soluble fraction A	Alcohol insoluble B	Recombined fractions A + B	Distilled water	Cc. N/20 H_2SO_4 added to 10 cc. juice	Original extract	Alcohol soluble fraction A	Alcohol insoluble B	Recombined fractions A + B	Distilled water
	pH	pH	pH	pH	pH		pH	pH	pH	pH	pH
0	5.85	3.89	6.26	5.61	6.30	0	5.85	3.93	6.24	5.60	6.25
1	6.33	5.16	6.95		11.30	1	5.47	3.16	5.70	5.29	2.41
2	6.83	7.44	7.66	6.53	11.55	2	5.18	2.64	5.29	5.04	2.16
3	7.25	8.86	8.60	7.25	11.69	3	4.94	2.40	4.98	4.84	2.04
4	7.76	9.48	9.22	7.86	11.78	4	4.74	2.22	4.75	4.64	1.95
5	8.35	9.96	9.64	8.33		5	4.56	2.11	4.53	4.48	
6	8.79	10.40	9.90	8.76	11.88	6	4.39	2.03	4.35	4.32	1.83
7	9.09	10.79	10.19	9.08		7	4.25	1.96	4.17	4.19	
8	9.34	11.06	10.35	9.33		8	4.10	1.91	4.01	4.05	
9	9.55	11.22	10.54	9.57		9	3.97		3.84	3.91	
10	9.73	11.36	10.73	9.76	11.98	10	3.85	1.82	3.68	3.76	1.72
11	9.92	11.46	10.91	9.92		11	3.71		3.52	3.64	
12	10.05	11.52	11.05	10.07		12	3.58	1.75	3.36	3.51	
13	10.24		11.17	10.23		13	3.44				
14	10.41	11.63	11.27	10.36		14	3.32	1.71	3.06	3.24	
15	10.58		11.36	10.50	12.04	15	3.21				1.64
16	10.73	11.70	11.43	10.65		16			2.79	3.00	
17	10.85			10.78		17	2.95	1.66			
18	10.98		11.54	10.87		18			2.57	2.79	
19	11.10			10.98		19					
20	11.18	11.79	11.62	11.07	12.09	20	2.65	1.61	2.40	2.62	1.58

The differences in the form of the titration curve for juice of seedlings in successive stages of development indicate differences in composition associated with increasing photosynthetic activity. The end of this period of change in the curve may be interpreted as the end of the seedling stage.

The progressive increase in buffer capacity during the maturation period is due to increasing concentration of the juice. The specific gravity increases regularly during this period, and is closely correlated with the corresponding "titratable acid" values. The initiation of these changes marks the beginning of the maturation period.

Heat-coagulable proteins play a very small part in the buffer system of the wheat plant as shown by the fact that their removal by boiling and filtering reduces the buffer action of the juice only slightly. The buffers remaining in boiled and filtered juice are soluble in either hot or cold 75-per cent. alcohol, partly soluble in hot 95-per cent. alcohol, but practically insoluble in cold 95-per cent. alcohol.

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LIPIDES AND THEIR ESTIMATION IN VEGETABLE TISSUES[1]

CHARLES E. SANDO

When plant tissues are extracted with ether or any of the other fat solvents, the extract contains among other things a large number of substances which yield fatty acids on hydrolysis. The commonest of these and perhaps the most important from a biochemical standpoint are the fats, fatty oils, waxes, sterol esters, phosphatides and cerebrosides. There are also associated with these classes of compounds in various extracts many other substances, some of which are the wax alcohols, sterols, essential oils, hydrocarbons, resins and coloring matters. Some of these accompanying substances are similar to the fats and other fatty acid compounds in possessing a greasy feel but they bear little or no resemblance from a strictly chemical standpoint. Notwithstanding this divergence in chemical makeup, many writers include them in a single large group. It is evident that such a grouping is based almost entirely on the common physical property of being soluble in one or more of the ordinary fat solvents.

Early investigators considered an ether extract of most tissues to consist mainly of neutral fats and other simple glycerides. Later, however, it was definitely established that certain other substances resembling the fats in their solubilities also were removed by ether. To these "fat-like" compounds, the term "lipoids" was applied and is still recognized in this sense by many notwithstanding the fact that the term was first suggested to denote only unsaponifiable substances and has since been extended to include in addition to the "fat-like" substance also the fats and other simple glycerides. Two other terms, "lipins" and "lipides," have also been proposed in connection with the substances under consideration. It will be seen from the following brief discussion of these terms that the nomenclature of the fats and fat-like compounds is still rather confusing.

The term "lipoids" was introduced nearly 70 years ago by KLETZINSKI (**77**) to denote unsaponifiable materials extracted from animal tissues by alcohol and ether. Later, OVERTON (**64**) used the same term to designate those tissue substances which were similar to the fats in being soluble in organic solvents such as ether, chloroform, and alcohol. Since OVERTON's time the term has been employed in several different ways. It has been used in a restricted sense to indicate only the phosphatides and cerebrosides. Reference to fats and lipoids have therefore been made on the assumption that these classes of substances properly belong to separate categories. Many have considered the word "lipoids" a convenient designation for a

[1] A review written at the request of the Committee on Methods of Chemical Analysis for the American Society of Plant Physiologists.

chemically heterogeneous group of substances which are characterized primarily by their physical property of common solubility in one or more of the ordinary fat solvents. LEATHES (**41**) looks upon the term as "a cloak for ignorance and an indefinable limbo into which any one can thrust anything of which he knows little or nothing, including often what is not a compound of any fatty acid at all." Recently, LEVINE (**47**) in proposing a new classification of fatty compounds soluble in ether or alcohol-ether and closely associated substances ignores the broader significance which had been attached to the term "lipoids" and limits its application to the sterols and essential oils.

The designation "lipins" was suggested by LEATHES (**42**) in 1910 for those derivatives of fatty acids which contain nitrogen but no phosphorus or carbohydrate group—an indefinite class of substances previously called "amidolipotides" by THUDICHUM (**87, 88**). Later, ROSENBLOOM and GIES (**73**) employed the term in place of "lipoids" as a group name for the fats and fat-like compounds yielding fatty acids on hydrolysis and also included under lipins a large number of diversified, non-fatty compounds and artificial products such as soaps, alcohols, sterols, cholates, chromolipins, triacetin and lead oleate. MCLEAN (**56**) has used the word "lipins" in a limited sense to embrace only the phospatides and cerebrosides which he considers substances of fat-like nature yielding on hydrolysis fatty acids or derivatives of fatty acids and containing in their molecule either nitrogen or nitrogen and phosphorus.

"Lipides" is the third generic name which has been proposed in the general nomenclature of the substances under consideration. It was first suggested and adopted in 1925 by the Committee on the Reform of the Nomenclature of Biological Chemistry at the International Congress of Chemistry meeting at Cambridge (**19**). It was recently used by BLOOR (**6**) in a modified classification in preference to "lipoids" as a group-heading for ether or alcohol soluble compounds yielding fatty acids on hydrolysis and certain derived substances such as fatty acids and sterols. Since this term has been used only in the broad significance to include both the fats (including oils and waxes) and the lipoids, as used in the restricted sense to denote the "fat-like" substances, it appears to be the best one suggested so far.

Various classifications of fats and "fat-like" compounds

A strictly chemical grouping of these substances has proved a difficult matter, especially in view of the diversity of the chemical relationships of the compounds. For purposes of comparison, the more important attempts to classify the fats and "fat-like" compounds are listed here.

THUDICHUM'S CLASSIFICATION, 1884 (87, 88)

Phosphatides or phosphorized principles:

A. **Mononitrogenized monophosphatides** (N: P:: 1: 1)
lecithins, kephalins, paramyelins, myelins.

B. **Dinitrogenized monophosphatides** (N: P:: 2: 1)
amidomyelins, amidokephelins, sphingomyelins.

C. **Dinitrogenized diphosphatides** (N: P:: 2: 2)
assurin.

D. **Nitrogenized phosphatide-sulphatides**
cerebrosulphatide.

E. **Nonnitrogenized monophosphatides**

Nitrogenized nonphosphorized principles:

A. **Cerebrosides**
phrenosin, kerasin.

B. **Cerebrinacids**
cerebrinic acid, spheracerebrin, etc.

C. **Cerebrosulphatides**

D. **Amidolipotides**
bregenin, krinosin.

ROSENHEIM'S CLASSIFICATION, 1909 (77)

Lipoids:

1. **Cholesterin** group
cholesterin, phytosterins, lipochromes, etc.
2. **Cerebro-galactosides**
phrenosin, kerasin.
3. **Phosphatides**
Monamino-monophosphatides (N: P:: 1: 1)
lecithins, kephalins, vesalthin.
Diamino-monophosphatides (N: P:: 2: 1)
sphingomyelin.
Triamino-monophosphatides (N: P:: 3: 1)
neottin.
Triamino-diphosphatides (N: P:: 3: 2)
Monoamino-diphosphatides (N: P:: 1: 2)
cuorin.

LEATHES' CLASSIFICATION, 1910 (42)

Substances entering into the composition of fats:

A. **The fatty acids**

B. **Glycerol and the glycerides**
fats and oils.

C. **Other alcohols and their fatty acid esters**
cetyl alcohol, myricyl alcohol, cholesterol, phytosterol, waxes, esters of cholesterol, etc.

D. **Phospholipins**
compounds of fatty acids containing N and P., *e.g.*, lecithin.
Galactolipines
compounds of fatty acids containing N and galactose, *e.g.*, cerebrone.

Lipines
compounds of fatty acids containing N but no P nor carbohydrate group, *e.g.*, **sphingosine.**

Bang's classification, 1911 (2)

Lipoids:

1. **Fats**
2. **Cholesterols**
3. **Phosphatides**
   - A. **Unsaturated phosphatides**
     - **Monaminomonophosphatides**
       lecithin, kephalin, paramyelin, vesalthin.
     - **Monaminodiphosphatides**
       cuorin, etc.
     - **Triaminodiphosphatides**
       sahidin, etc.
   - B. **Saturated phosphatides**
     - **Diaminomonophosphatides**
       sphingomyelin, aminomyelin, apomyelin, etc.
     - **Triaminomonophosphatides**
       neottin, carnaubon.
     - **Protagon**
4. **Cerebrosides**
   phrenosin, kerasin, cerebron, etc.

Cramer's classification, 1911 (12)

Lipoids (with the exception of the cholesterols):

1. **Phosphatides**
   N-containing fatty acid esters of glycero-phosphoric acid. Some may have the glycerol substituted by an unknown alcohol. Lecithin, kephalin, sphingomyelin, etc.
2. **Galacto-phosphatides**
   N-containing fatty acid esters of phosphoric acid, with galactose and alcohol groups. Carnaubon.
3. **Cerebrosides**
   N-containing fatty acid esters with galactose but no P. Cerebron, cerebrin, homocerebrin, etc.
4. **Phospho-cerebrosides**
   cerebrosides with P-containing groups. Protagon.

Rosenbloom and Gies's classification, 1911 (73)

Lipins, organic substances insoluble in concentrated saline solutions, soluble in hot alcohol or in warm ether or in both.

1. **Natural aliphatic lipins**
   - A. **Simple lipins**
     - a. **Fatty acids**
     - b. **Salts and esters of 1-A-a**
       soaps, waxes, fats and fatty oils.
     - c. **Alcohol** (mono- and di-hydroxy)
       cetyl alcohol, myricyl alcohol.

B. **Conjugate lipins**

a. **Proteolipins**

lecitho-protein like substances (may be mixtures).

b. **Glycolipins**

THUDICHUM'S cerebrosides.

c. **Phospholipins**

THUDICHUM'S phosphatides.

d. **Glyco-phospholipins**

phospholipins containing carbohydrate group.

2. **Natural carbocyclic lipins**

A. **Sterols**

natural terpeno-alcoholic derivatives, nonsaponifiable, and form esters, cholesterol, sitosterol, etc.

B. **Esterols**

natural terpeno-aliphatic waxes, *e.g.*, cholesterylpalmitate

C. **Cholates**

a. Cholic acids

b. Bile acids

3. **Natural lipins of undetermined constitution**

A. **Chromolipins**

B. **Miscellaneous lipins**

4. **Artificial lipins**

tri-acetin, lead oleate, etc.

MATHEW'S CLASSIFICATION, 1914 (54)

Lipins—constituents of protoplasm with greasy feel; soluble in alcohol-ether.

1. **Fats**

Fatty acids

2. **Fatty oils**

drying oils, semi-drying oils, non-drying oils.

3. **Essential oils**

volatile, generally odoriferous substances of oily and of varied chemical nature, being aldehydes, acids, terpenes, alcohols, etc.

4. **Waxes**

esters of sterols and fatty acids.

5. **Sterols**

alcohols, generally of the terpene group, solid at ordinary temperature; oxidation products are terpenic acids.

6. **Phospholipins** (phosphatides of THUDICHUM)

Mono-amino-monophospholipins, lecithin, cephalin.

Di-amino-monophospholipins

Mono-amino-diphospholipins

7. **Glycolipins** (cerebrosides of THUDICHUM), cerebron, phrenosin, etc.

8. **Sulpholipins**—not well characterized.

9. **Aminolipins**—not well characterized.

MACLEAN'S CLASSIFICATION, 1918 (56)

Substances in an ether or alcohol extract of a tissue:

A. **Neutral fat and fatty acids**

B. **Substances of varying chemical nature having no relation to fat** such as cholesterol and certain pigments.

Lipins: "fat-like" bodies, sometimes referred to as "lipoids"

C. **Phosphatides**

1. **Monoaminomonophosphatides** (N: P:: 1: 1), lecithin, kephalin.
2. **Monoaminodiphosphatides** (N:P::1:2), cuorin.
3. **Diaminomonophosphatides** (N:P:: 2:1), sphingomyelin.

D. **Cerebrosides**

phrenosin, kerasin.

LEVINE'S CLASSIFICATION, 1925 (47)

True lipins, compounds soluble in ether or alcohol-ether yielding on hydrolysis fatty acids and alcohol.

1. **Simple lipins,** compounds which yield on hydrolysis, fatty acid and glycerol or fatty acid and a monohydric alcohol of high molecular weight.
   a. **Fats**
   b. **Fatty oils**
   c. **Waxes**
2. **Conjugated lipins,** compounds which yield on hydrolysis, not only fatty acid and alcohol but some other complex such as sulphuric acid, phosphoric acid, monosaccharide, amino-acid, or some organic base like choline.
   a. **Phospholipins, lecithin,** cephalin, sphingomyelin.
   b. **Glycolipins,** cerebrin, phrenosin, kerasin.
   c. **Glycophospholipins,** jecorin.
   d. **Sulpholipins**
   e. **Sulpho-phospholipins**
   f. **Aminolipins,** bregenin.
   g. **Proteolipins**
   h. **Chromolipins**
3. **Derived lipins,** compounds other than phosphoric acid, sulphuric acid, amino acid or monosaccharide, obtained as a result of the decomposition of lipins.
   a. **Fatty acids**
   b. **Alcohols,** glycerol, myricyl alcohol, etc.
   c. **Organic bases,** choline, neurine, etc.

Lipoids, compounds not esters of fatty acid and alcohol, but which are closely associated with lipins and resemble them in their solubility in ether or alcohol-ether.

1. **Sterols,** cholesterol, phytosterol, etc.
2. **Essential oils**

BLOOR'S CLASSIFICATION, 1925 (6)

Lipides, substances having the following characteristics: (a) insolubility in water and solubility in fat solvents, such as ether, chloroform, benzol, (b) relationship to the fatty acids as esters, either actual or potential, (c) utilization by living organisms.

Simple lipides. Esters of the fatty acids with various alcohols.

Fats
Waxes

Compound lipides. Esters of fatty acids containing groups in addition to an alcohol and fatty acid.

Phospholipides, containing phosphoric acid and nitrogen; lecithin, cephalin, sphingomyelin.

Glycolipides, containing carbohydrate and nitrogen but no phosphoric acid; cerebrosides.

Aminolipides, sulpholipides, etc., not at present sufficiently well characterized for classification.

Derived lipides. Substances derived from the above groups by hydrolysis.

Fatty acids

Sterols, mostly high molecular weight alcohols, found in nature combined with fatty acids and which are soluble in the fat solvents; cholesterol, myricyl alcohol, etc.

It is not within the scope of this paper to discuss the relative merits of the various classifications, but it must be noted in passing that a great deal of confusion and difference of opinion exists in the literature as to what properly constitutes a single group of closely related fatty compounds and as to what terms should be used in their designation.

In the present paper, the use of the term "lipides" is preferred because of the fact that the several meanings which have been attached to the other two terms tend to create a state of disorder in an already difficult subject and therefore constitute an objection which does not apply in the case of "lipides." On the basis of chemical relationships, the BLOOR scheme appears more nearly to meet the requirements of a good classification than the others. The writer does not agree with BLOOR, however, in always considering the sterols as being derived from ester-like compounds.

According to BLOOR'S definition, lipides are water insoluble, ether or alcohol soluble substances which are either ester-like combinations with fatty acids or are capable of forming such combinations. These substances are separated into three main groups. The fats and waxes are considered as simple lipides, being esters of the fatty acids with various alcohols. The phospholipides, glycolipides and certain insufficiently characterized substances constitute the compound lipides or esters of fatty acids containing groups in addition to an alcohol and fatty acid. The free fatty acids and sterols represent the derived lipides, which are defined as substances obtained from the other groups by hydrolysis. For a thorough discussion of the various types of fatty compounds which are included in the foregoing classification and for their physical and chemical properties, one may refer to BLOOR'S original paper (**6**).

A great deal of confusion would be avoided if plant physiologists and biochemists in general would adopt the BLOOR scheme and designate as lipides those tissue constituents which fall under this classification. When obtained from tissues in the form of an impure extract by means of a fat solvent they could be reported as "crude lipides."

The estimation of lipides

Nearly all methods for determining lipides are based on the fact that these substances, or the fatty acids derived therefrom by saponification, are largely separated from the other tissue constituents by means of certain solvents which exert at least a partial preferential solubility for the fatty compounds. Petroleum ether, especially, and ether, to a less degree, are better suited for this purpose and therefore are used more extensively than other solvents for lipide estimation. While all lipides are more or less soluble in a number of so-called fat solvents, not all of these extract the lipides with equal facility (**37**) or extract the same non-lipide substances. With dry oat kernels (**5**), for example, if the total 15-hour ether extract is considered equivalent to 100, the quantities of material extracted by other fat solvents are as follows: petroleum ether 97.07, carbon tetrachloride 104.24, chloroform 109.78, acetone 112.71, benzene 113.15 and absolute alcohol 127.93. In this particular case petroleum ether, of all the solvents, removes the smallest quantity of extract, while absolute alcohol removes the largest. It should be recognized that in most instances the extract obtained from plants by the use of petroleum ether contains the least quantity of non-lipide substances. Ether, carbon tetrachloride, chloroform, and the other solvents are all known to extract increasing quantities of impurities such as resins, alkaloids, coloring matters and other organic substances. Regardless of the fact that petroleum ether is probably the more suitable solvent to use for the extraction of lipides in many cases, many plant physiologists use ether instead when extracting dried plant tissues. In fact, this has become the most generally used fat solvent in the laboratory. However, in the crude lipide extract obtained from many plant tissues by the use of ether the non-lipide substances may amount to more than the total weight of true lipides. This fact introduces a considerable error in case the extract is reported as "crude fat" or "lipoids" as is very often done by investigators working in agronomic and horticultural fields.

Next to the careful selection of solvent it is very important that attention be paid to the purity of that solvent. It is well known, for example, that impure ether, containing water and alcohol, will yield a greater quantity of extract than the pure anhydrous solvent yields. Such extracts are contaminated with more non-lipide substances than extracts obtained by the use of a pure solvent.

The foregoing statements are sufficient to emphasize the importance of the solvent in the extraction and estimation of lipides from vegetable tissues. On this account, it seemed desirable to include in this paper general data dealing with the common fat solvents[2], including a table of physical

[2] Compare also the following: **(53, 8, 97, 52)**.

constants. It must be remembered, however, that due to accompanying substances which exert an influence on their behavior, the solubilities of the pure lipides in pure solvents do not necessarily hold when lipides are extracted from tissues.

Lipide solvents

Petroleum ether (C_nH_{2n+2}).—Petroleum ether (b. p. 40–60°) readily dissolves most fats, oils, waxes, phospholipides, fatty acids and sterols; some of these even in the cold. Hydroxy acids and glycerides of these acids, for example, castor oil, are insoluble or nearly so in this solvent. Glycerides of the solid fatty acids are more difficultly soluble than those of the liquid acids. Pure tristearin, therefore, is only slightly soluble, but it becomes more so in the presence of soluble glycerides. Of the fatty acids, those of higher melting point are the least soluble. Hydrocarbons, essential oils, certain coloring matters to a slight extent, such as chlorophyll "a" and carotin, and a few other organic substances are soluble in petroleum ether. Despite the fact that this solvent dissolves certain non-lipide substances, it may be said to remove from plant materials the least quantity of these impurities in comparison with other solvents. This is particularly true with respect to coloring matters and compounds of a resinous nature.

Ethyl ether (C_2H_5–O–C_2H_5).—Practically all lipides are fairly readily soluble in ether with the exceptions of sphingomyelin and the cerebrosides. Fats are more easily soluble the higher the content of glycerides of unsaturated fatty acids and those of low molecular weight. Pure tristearin is difficultly soluble but is more so in the presence of other glycerides. Lecithin is generally considered soluble but its complete removal from plant tissues with ether, especially from leguminous seeds is difficult. The extraction of this and similar substances which are but slowly removed even on prolonged extraction is greatly facilitated by alcoholic pre-treatment (**7**). It is generally known that ether dissolves more non-lipide materials from plant tissues than petroleum ether. Among the soluble substances may be mentioned hydrocarbons, essential oils, coloring matter (including chlorophylls, carotinoids, free flavones and flavonols), many alkaloids, organic acids, resins and related compounds. Chibnall and Channon (**11**) state it has been their experience that the presence of a small amount of water in ether will allow a small but definite amount of inorganic matter and amino-compounds to go into solution. The same statement holds true with respect to carbohydrates and possibly tannins.

Carbon tetrachloride (CCl_4).—Carbon tetrachloride dissolves most lipides with the same ease as ether, but it probably removes more impurities (**70**). The observation of Schindelmeiser (**80**) that many alkaloids are soluble in cold carbon tetrachloride indicates that these substances would

TABLE I

TABLE OF CONSTANTS FOR PURE FAT SOLVENTS[3]

SOLVENT	BOILING POINT	SPECIFIC GRAVITY	VAPOR PRESSURE (mm. Hg.)		HEAT OF VAPORIZATION		SOLUBILITY	
			AT 20°	AT 30°	cal./gm.	K cal./mol.	SOLVENT IN WATER	WATER IN SOLVENT
Petroleum ether							At 22° = 0.227 per cent.	At 22° = 0.50 per cent.
pentane	36.3°	.6454$^{20}_{4}$	420.2	610.9	85.0			
n-hexane	68.6°	.6794$^{20}_{4}$	120.0	185.4	79.2	7.80	(Petroleum ether of sp. gr. = .6646)	
Ether	34.6°	.7191$^{15}_{4}$	538.0	914.0	84.5	6.26	At 20° = 1.22 per cent.	At 20° = 6.48 per cent.
							At 30° = 1.25 per cent.	At 30° = 5.04 per cent.
Carbon tetrachloride	76.7°	1.6319$^{20}_{4}$	91.3	141.1	46.4	7.14	At 20° = 0.08 per cent.	
							At 30° = 0.085 per cent.	
Chloroform	61.0°	1.4989$^{15}_{4}$	158.4	240.0	61.4	7.35	At 20° = 0.617 per cent.	At 20° = 0.10 per cent.
Trichlorethylene	88.0°	1.47$^{15}_{15}$	56.0	92.0	58.0		At 22°, 100 cc. of water dissolves 0.08 gm. or 0.5 cc.	
Carbon disulphide	46.3°	1.2922$^{20}_{4}$	298.0	717(40°)	86.7	6.60	At 22° = 0.218 per cent.	At 22° = 8.76 per cent.
Benzene	79.8°	.8799$^{20}_{4}$	75.0	118.0	94.9	7.41	At 25° = 0.113 per cent.	At 25° = 0.023 per cent.
Acetone	56.6°	.8125$^{20}_{4}$	184.8	282.7	125.0	7.26		
Alcohol	78.3°	.7093$^{15}_{4}$	44.0	78.06	216.5			

[3] Compiled from the following: (40, 97, 8).

be readily removed from plant tissues on continuous extraction with the hot solvent. For a more detailed account of the solvent properties, refer to BASKERVILLE and RIEDERER (**3**). BRYANT (**10**) claims carbon tetrachloride extracts the fats and similar substances in a much shorter time than is required for some of the other solvents. His results seem to indicate that this solvent removes in 2 hours the same quantity of fat (lipides) obtained in 16 hours by the use of ether and in 4 hours by carbon disulphide. WILEY (**94**) says "carbon tetrachloride would be a most desirable solvent, owing to the fact that it is not inflammable and that it dissolves nearly all the fats and oils with ease, if it were not that the last traces of it are removed with the greatest difficulty and also that in the presence of even minute traces of moisture it is partly decomposed into hydrochloric acid, which of course may act upon the fat and make it useless for further determinations."

CHLOROFORM ($CHCl_3$).—Chloroform is a good solvent for most lipides. However, it dissolves the same and probably more non-lipide substances than ether. The fact that it dissolves more substances than ether does is readily shown by extracting plant materials exhaustively with ether and then following with chloroform, in which case the chloroform will be found to remove a further appreciable quantity of soluble substances. SCHLESINGER (**81**) and ROSENFELD (**76**) use chloroform as solvent in lipide determination methods. In the ROSENFELD method, the material is first heated on the water-bath with alcohol, then extracted in a continuous extractor with chloroform. Finally the dry residue, obtained after the evaporation of the solvent, is extracted with absolute ether. KUMAGAWA and SUTO (**38**) have pointed out that the energetic solvent involved in the more efficient removal of lipides in this method is mainly the alcohol and not the chloroform.

CARBON DISULPHIDE (CS_2).—This solvent is a liquid of high solvent power for a wide range of substances. It readily dissolves oils, fats, waxes, resins, and many other organic substances. It has not come into general use for quantitative lipide estimation, probably because of its poisonous properties, its unpleasant odor and its liability to decomposition when used for the extraction of certain lipides.

BENZENE (C_6H_6).—Benzene is a good solvent for fats, oils, waxes, sterols and phospholipides. Cerebrosides are soluble in the hot solvent. Fatty acids in general are easily soluble and to a higher degree than in petroleum ether. Here also the solid fatty acids are less soluble than the liquid unsaturated ones. For example, oleic acid is soluble in all proportions, but stearic acid only to the extent of 0.22 parts in 1 of benzene at 23° C. Soaps are taken up in not inconsiderable quantities if free fatty acids or neutral oils are present. Benzene dissolves hydrocarbons, certain coloring matters, resins and many other organic compounds. Last traces of benzene are very difficult to remove from a lipide residue.

TRICHLORETHYLENE (C_2HCl_3).—This solvent dissolves most lipides very readily, but it also dissolves many other organic compounds, among which may be mentioned (**21**) benzoic, salicylic, and cinnamic acids, acetaldehyde, benzaldehyde, vanillin, camphor, alizarin, asparagine, glycine, caffeine and theobromine. Many of these non-lipide substances are doubtless left behind when the lipides in a sample are being determined by simple shaking with cold trichlorethylene and evaporation of an aliquot part of the fat solution.

ACETONE (CH_3–CO–CH_3).—Acetone has a wide range of solvent power for many classes of organic compounds. The fats, oils, and waxes are only slightly soluble in the cold, but readily soluble in the hot solvent. The cerebrosides and fatty acids are soluble, but lecithins and cephalins are practically insoluble. Sphingomyelin is insoluble or nearly so in cold but somewhat soluble in hot acetone. Hydrocarbons, sterols, many resins, coloring matters, certain glucosides, tannins and other compounds are all more or less soluble in this solvent. Its solvent action is too general to permit its use for quantitative work. It has been recommended in place of alcohol to extract the lipides where the residue obtained is subsequently to be extracted with ether or petroleum ether to separate impurities.

ALCOHOL (C_2H_5OH).—Alcohol is a most excellent and energetic solvent for many classes of organic compounds. In this respect it may be said to possess a wider range of solvent power for plant constituents than any other solvent with perhaps the single exception of water. It is known to extract many compounds belonging to the following classes: lipides, carbohydrates, alkaloids, glucosides, tannins, saponins, resins, organic acids, bases, proteins (a few are soluble), coloring matters, etc. With respect to fats, oils, and waxes it may be stated that they are only slightly soluble in cold, but are more readily dissolved in the hot solvent. The solubility of fats and oils varies with the nature of the combined fatty acids. Glycerides of lower, unsaturated fatty acids being more soluble than those of the higher, saturated ones. LEWKOWITSCH (**48**) points out that the solubility of most fats and oils in absolute alcohol at 15° does not exceed 2 per cent. while in 95 per cent. alcohol the solubility is still less. Castor oil is a notable exception, being readily soluble in all proportions at ordinary temperature. Palmitin, stearin, and olein are almost insoluble in 91 per cent. alcohol. At 35° myristin is little soluble, while laurin is easily soluble. At higher temperatures the solubilities increase. The presence of free fatty acids also influences solubility, the presence of 50 per cent. making solution complete. Free fatty acids are soluble in alcohol, although from palmitic upwards all are sparingly soluble. For example, oleic acid and most unsaturated acids are readily soluble, but only 9.3 parts of palmitic and 2.5 parts of stearic acid are soluble in 100 parts of alcohol. Cerotic acid is almost insoluble at 20°,

but soluble in boiling alcohol. The majority of oils and fats are soluble in absolute alcohol. In the use of alcohol at its boiling point for the removal of lipides, there is some liklihood of loss due to decomposition of the more sensitive lipides. The use of alcohol under reduced pressures as a means of extracting lipides from vegetable tissues without decomposition is well worthy of consideration. The possibility of the solvent reacting with the dissolved fatty acids at the ordinary boiling point of alcohol must not be overlooked in the use of this solvent to extract plant materials. HOLLAND (**30**) claims boiling with alcohol causes a drop in the neutralization number of fatty acids, particularly stearic and myristic acid. It was observed by EMERSON and DUMAS (**17**) that 0.3 per cent. of palmitic acid was esterified in the short time necessary for the solution of this acid in alcohol on the water-bath and the evaporation of the alcohol. Of the phospholipides, cephalin is characterized by its insolubility in alcohol. Sphingomyelin is only slightly soluble in the cold, but soluble in hot alcohol. Lecithins are soluble. The glycolipides (cerebrosides) and sterols are readily soluble in hot alcohol.

CRUDE LIPIDE DETERMINATION METHODS

The principles involved in the extraction of lipides are practically the same whether one is dealing with animal or plant tissues. We may therefore conveniently separate the more important methods for the estimation of lipides in both classes of material into two general groups.

FIRST GROUP.—In the first group may be placed those methods which are based on the removal of lipides from the tissues in a condition as little altered as possible. The methods in this group may be subdivided into (a) those based on the direct extraction of the material with a fat solvent or a combination of solvents, and (b) those in which the material is subjected to a mild pretreatment such as pepsin digestion, or treatment with dilute acid or alkali, followed by extraction with a fat solvent.

The more important methods under "a" are those of shaking with ether, petroleum ether, or trichlorethylene according to LOGES (**51**), SCHÜTTE (**83**), MONHAUPT (**57, 58, 59**), GRIMME (**22**), NEUMANN (**61, 62**, *cf.* also PHILLIPS, **68**) and GROSSFELD (**23, 24, 25, 26**); the ether extraction methods of SOXHLET (**85**, *cf.* also BÖMER, **9**) (with modifications adopted as the "Official method" by the Association of Official Agricultural Chemists (**96**)), LEHMANN (**44**) and VÖLTZ (**91**); the petroleum ether extraction methods of GLIKIN (**20**), LEPPER and WATERMAN (**46**); the carbon tetrachloride extraction method of BRYANT (**10**); the alcohol-ether, alcohol-petroleum-ether, or alcohol-chloroform methods of BOGDANOW (**7**), FRANK (**18**), VOIT (**90**), NOEL-PATON (**63**), HERTWIG (**29**), ROSENFELD (**74, 75, 76**); and the modified

Koch method (applicable to fresh tissues (**34, 35, 36**)) (*cf.* also Appleman (**1**), Culpepper, Foster and Caldwell (**13**), Jones (**32**), Walster (**92**), and Schertz (**79**)).

Under "b" are placed the acid-pretreatment methods of Pflüger (**67**), Palmquist (**66**), Polenske (**69**), Weibull (**93**); the ammoniacal-alcohol method of Rask and Phelps (**71**); and the pepsin-digestion method of Dormeyer (**15, 16**) (*cf.* also Schlesinger (**81**), Beger (**4**), Müller (**60**), and Diesselhorst (**14**)).

Second group.—In the second group may be placed those methods which are based on the extraction of the fatty acids produced on saponification of the lipides originally present in the tissues. This group includes the methods of Lieberman and Székely (**49, 50**) (*cf.* also Leathes (**43**), Rosenthal and Trowbridge (**78**), Kumawaga and Suto (**37**), *cf.* also Kumawaga (**39**), Inaba (**31**), Schimidzu (**84**), Lemland (**45**), and Terroine, Lepage, Véchot and Wolff (**86**), and of Rather (**72**)).

Not all of the methods included in the two foregoing groups have been employed for plant tissues. It is therefore not known whether they may be used without further modifications to meet the special needs of plant materials. Of those methods which are applicable to vegetable tissues only the three following will be discussed in detail.

1. Official method of the Association of Official Agricultural Chemists (96)

Abstract of method.—Two or more grams of properly dried and ground material are extracted for 16 hours with anhydrous ether. The extract, after evaporation of the ether, is dried for 30 minutes at the temperature of boiling water, cooled in a desiccator and weighed. The drying is continued for 30-minute periods until the weight of the residue remains constant.

Details of operation.—For the extraction of lipides from solid plant materials, according to the "Official method," the Soxhlet apparatus or one of its many modifications is generally used, although any of the numerous types of continuous extractors[4] which are now available may serve equally well. The quantity of dried material to be extracted will vary from 2 to 10 grams or even more, depending upon the percentage of fatty substances contained therein. The sample, carefully selected for uniformity, should be dried, preferably at 60° in a partial vacuum, and finely ground.[5] The accurately weighed substance is placed either in a Schleicher and

[4] For a discussion of automatic devices for the extraction of powdered material see Wiley (**95**) and Palkin and Walkins (**65**).

[5] See recommendations of Committee on Methods of Chemical Analysis for American Society of Plant Physiologists (**89**).

Schüll fat-free paper thimble, in a Schott and Company "glass filter" (carried by the Empire Laboratory Supply Company) or in a porcelain cup (**27**) and covered with a fat-free paper or cotton. The thimble and sample are placed in the extractor[6] and elevated, if necessary, by means of a glass tube of sufficient heighth so that the surface of the material is about 1 cm. under the top of the siphon tube. This adjustment insures a more complete extraction of the sample.

The flask, containing several glass beads to prevent bumping of the solvent during extraction, is thoroughly dried by heating, cooled over sulphuric acid in a desiccator, and weighed with the aid of a counterpoise of nearly the same size and shape. A portion of the solvent, about 50 to 150 cc., is poured into the flask, which is then connected with the extractor holding the sample. A further quantity of solvent is carefully poured over the material in the extractor until the height of the liquid nearly corresponds to the level of the top of the siphon tube. A tube containing anhydrous calcium chloride to prevent absorption of water by the solvent is attached to the top of the condenser.

The solvent is boiled during the period of extraction, either on an electric hot-plate or on a steam bath. The vapors of the solvent liquify in the condenser, the condensate flows into the extractor, penetrates the material and dissolves the lipides. When the liquid reaches the top of the siphon tube, it should automatically return to the flask. With some materials it is desirable to discontinue the extraction after the greater portion of lipides has been extracted and to reduce the sample to a finer state for a second and final period of extraction. The material, after drying to remove traces of the solvent, is ground in a mortar with 1/2 to 2/3 of its weight of pure quartz sand and the finely ground mixture quantitatively replaced in the thimble. Final traces in the mortar are obtained by removing with fat-free cotton, which must be added to the main sample in the thimble. The extraction is then continued as before. In some plant tissues the removal of lipides is nearly complete in 4 to 6 hours, in others 16 hours or longer are required. No definite time limit can be prescribed in all cases. This must be experimentally determined. In general, it may be stated that the size of particles and degree of porosity of sample are important factors influencing the rapidity and completeness of the extraction with any particular solvent.

Removal of the solvent from the extraction flask upon completion of the extraction is easily performed by using the apparatus itself for the distillation. After removal of the sample and glass tube, the boiling is continued until most of the solvent is distilled into the extractor. If necessary the solvent is siphoned off into a second container and the process continued

[6] Directions indicated apply to the Soxhlet apparatus.

until nearly all of the liquid in the flask has been evaporated. The final portions of solvent must be evaporated on a steam bath, care being exercised to prevent any spattering of the contents of the flask. Last traces of the solvent may be eliminated with a stream of pure dry carbon dioxide.

The residue in the flask is dried for 30 minutes at the temperature of boiling water, cooled over sulphuric acid in a desiccator and weighed. The drying is continued for 30-minute intervals, the flask being cooled and weighed each time, until the weight remains constant. If the material is very hygroscopic it may be necessary to dry at room temperature over calcium chloride in a vacuum desiccator until constant weight has been attained. If exactly 10 grams of dried substance are used as the original sample, the weight of the residue multiplied by 10 gives directly the percentage of fatty residue. This is sometimes reported as "fat" or "crude fat." It would be more nearly correct, however, to express the results as "crude lipides" or "ether extract."

General remarks.—When considering the ether extraction of dried samples according to the "Official method," it should be borne in mind that a portion of the lipides present in the fresh tissues inevitably suffers partial decomposition during desiccation. If the drying is carried out at the temperature of boiling water, and with exposure of the material to air, changes occur due to the partial oxidation of compounds containing unsaturated fatty acids. Such changes are minimized but not entirely eliminated if the drying is accomplished at reduced pressures or in an atmosphere of neutral gas.

Another error in the ether extraction method is due to the varying quantities of impurities which are removed from the tissues along with the true lipides and reported as such. The determination of lipides in cacao products furnishes an illustration. Here the use of ether results in the extraction of significant quantities of the alkaloid, theobromine. Other impurities such as hydrocarbons, coloring matters, and resin compounds are sources of error. Many of these may be determined and corrected for, where the quantity of residue is sufficient to justify an examination for unsaponifiable matter. In plant physiological work one is often limited with respect to quantity of available material, consequently the residue obtained from an ether extraction is usually too small to attempt further examination. In such cases it is desirable to have the residue represent as pure an extract of lipides as possible.

It is generally recognized that a small loss of lipides occurs through incomplete extraction. A portion of the lipides either becomes firmly entangled with carbohydrate and protein matter during the drying of the original sample, or the solvent is unable to penetrate the material properly

within a reasonable length of time. That the removal of ether soluble constituents is nearly always incomplete was shown by PFLÜGER (**67**) and by DORMEYER (**16**) in the case of dried animal materials. The difficulties experienced in removing lipides from plant tissues are not as serious as in the case of animal materials, since it is well known that lipides are held more tenaciously in animal tissues. SCHULZE and STEIGER (**82**) and MAXWELL (**55**) reported that phospholipides (phosphatides) can be completely removed from seed material only if the ether extraction is followed by successive extractions with alcohol at 60°. That an ether extraction of plant materials may be incomplete has also been pointed out by RATHER (**72**), HERTWIG (**28**) and by RASK and PHELPS (**71**).

2. MODIFIED KOCH METHOD[7]

ABSTRACT OF METHOD, FIRST PROCEDURE.—A sample of fresh material, weighing from 50 to 150 grams, is introduced into a sufficient quantity of boiling 95 per cent. alcohol to insure a final concentration of at least 80 per cent., after allowance for the original water content of the tissue. The mixture is boiled 15 minutes. The cold alcoholic liquid is filtered through extraction cups, and the residue exhaustively and successively extracted with alcohol, ether, and again with alcohol. The first alcoholic preserving filtrate and the combined alcohol and ether extracts are added together and evaporated to dryness. The residue is then thoroughly extracted with anhydrous ether. The extract, after the evaporation of ether, is dried to constant weight. The dried residue represents "crude lipides."

DETAILS OF OPERATION.—The fresh plant material to be analyzed is collected, rapidly cut into small pieces or otherwise reduced to finely divided state and thoroughly mixed to insure uniformity. Immediately after the material has been mixed, 50 to 150 gram duplicate samples are quickly and accurately weighed out and introduced into sufficient redistilled 95 per cent. boiling alcohol to bring the final concentration to at least 80 per cent., after allowance for the water originally present in the tissue. Boiling is continued for 15 minutes to arrest enzyme activity. Preserved in this manner, the material may be stored several months before the analytical work is begun, although it might be advisable to reheat the samples at least once or twice during the storage period.

[7] The original KOCH method was first employed in the analysis of animal tissues by WALDEMAR KOCH (**34, 35, 36**). Later F. C. KOCH, University of Chicago, introduced changes which were embodied in his unpublished student outline for the analysis of tissues. Students of plant physiology have made further modifications (**1, 13, 32, 92, 79**). The modified KOCH method provides for the separation of the constituents of tissues into a number of fractions which are separately examined. In the present paper discussion is confined to the so-called "lipoid" fraction.

For the extraction of the alcohol-preserved material, the insoluble residue is quantitatively transferred to Schleicher and Schüll fat-free paper extraction thimbles or to porcelain cups which are perforated on the bottom and fitted with filter-paper. If paper thimbles are used they may be placed upright in funnels in such a way that the liquid drips through the cups into flasks. The procelain cups are suspended by clamps over the flasks or beakers to accomplish the same purpose. In transferring the contents of the containers into the cups, the greater part of the material is poured into a beaker and the supernatant liquid from this used to transfer adhering matter from the container to the cup. The whole sample is then carefully removed to the cups, the last trace of substance being washed out with a jet of hot 95 per cent. redistilled alcohol. The alcoholic filtrate is set aside to be added later to the other portions of the extract.

The well-drained material in the extraction cup is covered with fat-free cotton or filter-paper, placed in a continuous extractor and extracted 4 to 12 hours with 95 per cent. alcohol. The alcoholic extraction is followed with an ether extraction for a period of 1–12 hours. Following the ether extraction the sample is freed of ether and powdered as finely as possible in an agate or glass mortar or small coffee mill. The powdered material is then carefully collected and replaced in the apparatus and extracted further for a period of 12 to 24 hours with redistilled 95 per cent. alcohol. The several alcohol and ether extracts including the original alcoholic preserving filtrate are now combined. In the case of the ether extract most of the solvent is removed by evaporation and the residue transferred to the alcoholic portions with aid of hot alcohol followed by a little hot water. Any solid matter adhering to the flask after this treatment may be removed with the aid of a little chloroform and finally hot water. The combined extracts are then evaporated to small bulk as rapidly as possible, without overheating, on the steam bath, quantitatively transferred to an Erlenmeyer flask, and the evaporation continued until all of the liquid is driven off. Evaporation should be hastened by the use of a suction tube extending into the mouth of the flask and by adding absolute alcohol from time to time. The residue is finally dried over calcium chloride in a vacuum desiccator. At frequent intervals the desiccator should be filled with dry air and again exhausted.

The transfer to the Erlenmeyer flask is performed to facilitate subsequent extraction with ether of the dry residue. This extraction may be carried out by adding successive quantities of anhydrous ether to the residue, heating just to boiling on an electric hot-plate with stirring, and quickly filtering the separate portions of ether through an ashless filter-paper into a weighed flask. The filtered extract is evaporated to dryness

on the steam bath and the residue dried to constant weight at the temperature of boiling water as in the "Official method." From the weight of the residue so obtained the percentage of "crude lipides" in the original sample may be calculated. A moisture determination must be made on a duplicate sample of the original material if a dry weight calculation is desired.

ABSTRACT OF METHOD, SECOND PROCEDURE.—The sample is preserved in 80 per cent. alcohol as described under the first procedure. The insoluble residue, after separation from the alcoholic preserving liquid, is successively extracted with alcohol, ether, water and again with alcohol. The combined extracts are evaporated and the residue made into an emulsion with water. The aqueous emulsion is treated with chloroform and dilute hydrochloric acid. The mixture is well shaken and allowed to remain in a cool place until the aqueous portion becomes clear. This is drawn off through a filter. The lipide portion remains dissolved in the chloroform layer or as insoluble matter adhering to the walls of the flask and to the filter-paper. These three separations are brought together with aid of hot alcohol and the entire volume of liquid evaporated to dryness in a weighed flask, or made up to definite volume with hot alcohol after the evaporation of the chloroform, and aliquot portions taken for dry weight determination.

DETAILS OF OPERATION.—Following the ether extraction as described under the first procedure, the insoluble residue is finely ground in an agate or a glass mortar or coffee mill. The powdered material is transferred to a flask and heated on the steam bath with about 100 cc. of water. Sufficient 95 per cent. alcohol is then added to bring the final concentration of alcohol to about 85 per cent. and the heating continued for another period. The mixture is then filtered through paper extraction thimbles, the residue being quantitatively transferred from the flask to the thimbles and extracted continuously with fresh 95 per cent. alcohol for 12 to 24 hours. The united alcohol, ether and water extracts are then evaporated on the steam bath at 75° to a syrupy consistency.[8] The residue is taken up with water and the aqueous emulsion is transferred to a glass-stoppered mixing cylinder or a volumetric flask having a capacity of one liter for each 100 grams of the sample. To wash adhering substances from the evaporation dish, small portions of chloroform are used which are added to the emulsion. The total volume of these washings should not amount to more than 20 or 25 cc. The flask or cylinder containing the emulsion is then filled to the mark with aqueous hydrochloric acid of such strength that the final volume

[8] Certain modifications are necessary from this point on if it is the intention to make determinations other than lipides on the same material, as outlined in the original method. For these consult CULPEPPER, FOSTER and CALDWELL (13).

of liquid will contain 2 or 3 cc. of concentrated acid for each 250 cc. The mixture is vigorously shaken at frequent intervals for 2 hours, then placed in an ice-box for 24 hours or until the aqueous layer has separated and become quite clear. This is drawn off by gentle suction through a long tube of small bore. By exercising great care, the aqueous solution can be separated almost completely from the chloroform layer. Should any solid matter be drawn off, it should be collected by filtering through a dry ashless filter-paper and washed with a little water. The lipide matter will be found in the chloroform layer and adhering to the walls of the precipitation flask and to the filter-paper. The tip of the latter is perforated and any solid matter adhering to the paper washed into the precipitation flask with a jet of hot 95 per cent. alcohol. The contents of the flask are then quantitatively transferred to a weighed flash and evaporated to dryness on the steam bath. The residue is dried to constant weight at the temperature of boiling water, or else at room temperature in a vacuum desiccator over calcium chloride. This represents the total quantity of "crude lipides." (The method of determining the lipide fraction by separating the aqueous layer from the chloroform fraction as described is not applicable to all materials. In such cases it is necessary to follow the procedure indicated in KOCH's student outline for the analysis of tissues to which reference has previously been made). In many cases it is desirable to determine in addition, the lipide phosphorus, lipide nitrogen and other constituents. For this purpose, the residue is dissolved in hot 95 per cent. alcohol and transferred to the volumetric flask. Ordinary redistilled alcohol is then added until the flask is about half full and then absolute alcohol to about two thirds full. The mixture is heated with gentle agitation on the steam bath until solution is uniform throughout, when the flask is filled to the mark with hot 95 per cent. alcohol. Aliquot portions are collected immediately for the various determinations, care being taken that the pipette for removing the liquid is first heated to the proper temperature in an oven or over a water-bath. Another modification which may be considered as a means of separating the lipide material from the other active ingredients is that of shaking the water emulsion of the alcohol-ether soluble material with pure ether at least four times or until a fresh ether extract no longer contains lipide material. The combined ether extracts are then shaken with distilled water to remove traces of water-soluble constituents. The ether extract is finally evaporated to dryness and the residue brought to constant weight as previously indicated.

GENERAL REMARKS.—The fact that the modified KOCH method makes use of fresh instead of dried material is the chief point in its favor. If the sample is heated immediately with alcohol as described there is very little

likelihood of autolytic changes. Partial oxidation of certain unsaturated compounds may go on to a slight degree even with hot alcohol treatment, but the error from this source is probably much less than in any method where it is necessary to dehydrate the sample by exposure to heat and air.

Another distinct advantage of the method is the provisions which are made for the systematic extraction and separation into definite fractions for the determination of other substances, in addition to the lipides, from the same material. These determinations have been discussed elsewhere.

The use of both alcohol and ether as the first stage in the removal of lipides is perhaps preferable to the use of ether alone, since it is generally recognized that extraction with ether, no matter how prolonged, fails to completely separate all of these substances, especially the phospholipides, from the other tissue constituents such as the carbohydrates and proteins. The incomplete extraction is particularly evident with material high in starch. LEATHES (**43**) states in referring to the estimation of fats that "the use of the alcohol for the first stage of the extraction has the advantage of removing water, and leaving the tissue in a condition to give up its fat more completely to ether; but, besides this, alcohol seems effectually to liberate fat from cells in a way not clearly understood, possibly counteracting the tendency for some fatty substances to adhere to surfaces in the presence of water, much as alcohol will remove a dye that has adhered to charcoal suspended in water." One objection to the use of alcohol is the fact that it simultaneously removes along with the lipides most of the soluble carbohydrates which, unfortunately, interfere to a certain extent with the subsequent direct extraction of the syrupy residue with ether. This difficulty is not encountered in the second procedure in which the fatty compounds are emulsified and then separated from other constituents with chloroform and dilute hydrochloric acid. The use of dilute hydrochloric acid at this stage does not appear to have any hydrolytic effect on the lipides (**33**), but the whole second procedure is rather lengthy and bothersome.

3. KUMAGAWA-SUTO METHOD

ABSTRACT OF METHOD.—The fresh or dried plant material, or an alcoholic extract thereof, is saponified several hours with caustic soda solution. The mixture is then neutralized with acid, cooled, and thoroughly shaken out with ether to remove the fatty acids formed. The filtered ether extract is evaporated and the residue again taken up in absolute ether. The dry residue from this extract is finally dissolved in petroleum ether, the solvent evaporated, and the residue dried to consistent weight at 50° in a vacuum, cooled and weighed. This represents total crude fatty acids. A determination of unsaponifiable matter is made on this residue and the weight so

obtained subtracted from the total crude fatty acids, which gives the weight of true fatty acids. This figure multiplied by the factor 1.046 gives the approximate weight of the corresponding neutral glycerides.

DETAILS OF OPERATION.—Either of two procedures may be followed in the method, (a) direct saponification of the material, and (b) alcoholic extraction with subsequent saponification of the alcohol extract. For direct saponification the material may be either fresh or in powder form. A sample of fresh material, weighing from 5 to 20 grams, is treated with 7 to 8 cc. of strong sodium hydroxide solution (sp. gr. = 1.5). If 5 grams are used, an additional 14 cc. of water are added. With 10 grams, 10 cc. of water are added, while above 20 grams it is unnecessary to make any further additions of water, the quantity of moisture present in the original tissues being sufficient. With dried material, 2 to 5 grams are treated with 25 cc. 5 N. sodium hydroxide solution (20 grams per 100 cc. of water).

The material and alkali in a 150 to 200 cc. beaker are placed on a boiling water-bath and covered with an open bell jar. At the end of 2 hours the warm mixture is quantitatively transferred to a 250 cc. separatory funnel. The last traces are removed from the beaker with 2 or 3 washings with small quantities of warm water. To the mixture in the separatory funnel, 30 cc. of a 20 per cent. hydrochloric acid solution (sp. gr.-= 1.1) are slowly added, with cooling. When thoroughly cool, the acid liquid is vigorously shaken with successive portions of ether. For the first shaking, 70 to 100 cc. of ether are used. The second and third require only 5 to 10 cc. for each shaking. The aqueous layer is drawn off each time and the ether layer collected in a beaker. During the foregoing shaking operations, a precipitate forms in the funnel at the point of partition of the two layers. This precipitate is dissolved in 5 cc. of N. sodium hydroxide solution. The alkaline solution is shaken with 30 to 50 cc. of ether. Then the first aqueous acid liquid is added and the whole vigorously shaken. The separated ether layer is added to the other ether portion and the united extracts evaporated. The dry residue is then redissolved in absolute ether. The ethereal solution is filtered through a specially constructed asbestos filter (**39**) and evaporated to dryness. The residue is dried at 50° for 1 to 2 hours. Drying longer than this period should be carried out in a vacuum. The residue contains, besides fatty acids, also hydrocarbons, sterols, coloring matters and other substances. Some of these impurities are removed at this stage. To the residue while still warm 30 to 40 cc. of petroleum ether are added and gently rotated. The beaker is then covered with a watch glass and set aside ½ to 1 hour to permit the settling of resinous matter. The fatty acid solution is filtered through asbestos which is well washed with petroleum ether. The filtrate and washings are collected in a weighed

100 cc. beaker and evaporated. The residue is dried to constant weight at 50°, preferably *in vacuo*. This weight represents crude fatty acids.

It is particularly important that care be taken to sufficiently dry the residue from the ether extract before extraction with petroleum ether, in order to obtain the fatty acids in pure colorless form. The ether used for the last extraction, as well as the petroleum ether, should be pure and anhydrous. The time for drying the residue at 50° must not exceed 1 to 2 hours unless the drying is carried out under reduced pressure.

With many plant materials, especially those rich in starch and other polysaccharides, it is necessary first to make an alcoholic extract (b) before proceeding with the saponification of the lipides. The sample is extracted with absolute alcohol in the KUMAGAWA and SUTO hot extractor (**39**) until all but traces of lipides have been removed. After the addition of 7 to 8 cc. of a strong sodium hydroxide solution (sp. gr. = 1.5) to the alcoholic extract, the mixture is boiled under a reflux condenser for an hour. The alcohol is then allowed to evaporate, the saponification continuing meanwhile. When the alcohol has been removed, the residue is taken up with a little warm water and quantitatively transferred to a separatory funnel. The fatty acids are separated as described under the direct saponification procedure (a).

The residue of the original material which has been extracted with alcohol may still retain a small quantity of unextracted lipides, but in most cases this is too small to be significant. It may be recovered, however, by treating the residue essentially according to the direct saponification procedure (a), the only modification being that after neutralization of the saponification mixture the starch is hydrolyzed with strong acid to liberate any retained lipides.

To separate unsaponifiable matter from the final residue obtained as described in either method, the residue is redissolved in petroleum ether and introduced into a separatory funnel. To dissolve completely the residue and to transfer the solution quantitatively from the beaker to funnel requires 50 to 70 cc. of petroleum ether. Thirty to 40 volumes of N/5 nearly absolute alcoholic potash are added to the solution in the funnel which is then well shaken once. A clear solution results. A quantity of water is added to this equal to the volume of alcoholic potash used, whereby a separation occurs with the 50 per cent. alcoholic liquid as the bottom layer and the petroleum ether solution as the top layer. The unsaponifiable substances remain in the top layer, while the soaps go to the dilute alcoholic layer. After separation of the solution, the dilute alcoholic layer is again extracted with 30 to 50 cc. of new petroleum ether. After evaporation of the petroleum ether from the combined shakings the residue is freed from the small traces of fatty acids which it contains. This separation is accom-

plished by dissolving the residue in a little absolute alcohol, adding 0.5 to 1.0 cc. N/10 nearly absolute alcoholic soda, evaporating on the water-bath, drying the residue 15 to 30 minutes at 100° and then extracting with hot petroleum ether. The petroleum ether extract is filtered through asbestos into a weighed flask, evaporated and dried to constant weight at 100°. This weight represents total unsaponifiable matter. It must be subtracted from the weight of total crude fatty acids previously obtained to find the quantity of true fatty acids. The weight of true fatty acids multiplied by the factor 1.046 gives the weight of the neutral glycerides.

The above-described method of KUMAGAWA and SUTO has been modified by LEMLAND (**45**). Very briefly, the LEMLAND procedure consists in extracting the material 8 hours in the KUMAGAWA and SUTO extractor with alcohol, evaporating and drying to constant weight *in vacuo*, saponifying the residue, extracting and weighing the unsaponifiable matter, acidifying and extracting the residual soap solution and drying to constant weight and weighing the fatty acids. According to TERROINE, LEPAGE, VÉCHOT and WOLFF (**86**) this method is directly applicable to vegetable products provided they have been finely ground before extraction.

GENERAL REMARKS.—To convert numbers representing the weight of fatty acids into numbers representing the weight of neutral fat, KUMAGAWA and SUTO employ the factor 1.046. This factor is obtained by assuming that on saponification an equal quantity of each of the three fatty acids, oleic, stearic and palmitic acids, is obtained from corresponding triglycerides. The following, which is taken from KUMAGAWA and SUTO (**37**) indicates how the factor was obtained:

Molecular weight of triolein	884.8
Molecular weight of tristearin	890.9
Molecular weight of tripalmitin	806.8
average	= 860.8
(oleopalmitostearate)	
Molecular weight of oleic acid	282.3
Molecular weight of stearic acid	284.3
Molecular weight of palmitic acid	256.3
total	= 822.9
(oleopalmitostearic acid)	

$$\frac{\text{Oleopalmitostearate}}{\text{Oleopalmitostearic acid}} = \frac{860.8}{822.9} = 1.046 = \text{factor}$$

Strictly speaking, not all of the insoluble fatty acids, as determined by saponification, are derived from simple triglycerides, nor are the quantities of these fatty acids always present in equal proportions. Furthermore,

fatty acids other than the three mentioned may occur in the extract. In many cases an appreciable percentage of the total fatty acids may be obtained by hydrolysis of waxes and phospholipides which contain different percentages of fatty acids than do the simple glycerides or fats. These are sources of errors which probably cannot be overcome since it is impossible to know definitely the relative proportions of different classes of lipides giving rise to fatty acids on saponification.

In this method, the volatile fatty acids are lost and only the high-molecular fatty acids are determined. KUMAGAWA and SUTO point out that in the saponification method of LIEBERMANN and SZÉKELY much too great a quantity of low fatty acids are determined, and furthermore about 9 per cent. of the high-molecular acids escape determination.

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A STUDY OF CERTAIN CONSTITUENTS OF THE LEAF AND THEIR RELATION TO THE BURNING QUALITIES OF TOBACCO[1,2]

D. E. Haley, E. S. Nasset, and Otto Olson

Introduction

The term "burning qualities" as applied to smoking tobacco, usually includes several different elements. Of these, according to Garner (**9**, p. 8), the fire holding capacity, the evenness and completeness of combustion, and the character of the ash are of most importance. A knowledge of the factors influencing the burning qualities of cigar-leaf tobacco is of considerable importance, both from the standpoint of the producer and the manufacturer, as the value of tobacco for cigar manufacture is based almost wholly upon these qualities. For this reason, therefore, considerable investigational work has been conducted upon this subject, and, as a result, it is now known that several factors, especially those of a chemical nature, influence the "burn" to a considerable extent.

Review of previous investigations

Schlösing (**23**) was one of the first investigators to study the chemical factors affecting the burning qualities of tobacco. He treated tobacco leaves with various salt solutions and found that potassium salts of various organic acids were decidedly beneficial. He attributed their effects to the tendency of these salts to swell on heating, thus exposing more surface of combustible material.

By impregnating filter-paper with solutions of various salts, Nessler (**20**, p. 73) found that the sulphate and carbonate of potassium increased the fire-holding capacity to a marked degree. He also observed that potassium acetate promoted combustibility in a manner similar to the carbonate, although this salt does not possess the power to swell when heated, at least to a marked degree, therefore, Schlösing's interpretation of the catalytic effect of potassium salts of organic acids was probably not correct.

In analyzing the ash of good-burning tobacco, van Bemmelen (**24**) always found a greater quantity of potassium present as the carbonate and lesser quantities as the chloride and sulphate.

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[2] This investigation was conducted in cooperation with Dr. W. W. Garner, of the U. S. Bureau of Plant Industry, Office of Plant Nutrition and Tobacco Investigations, and Professor F. D. Gardner, Department of Agronomy of the Pennsylvania State College.

MAYER (**16**) studied the influence of organic and inorganic substances on the capacity of filter-paper to glow or burn with a flame. The results obtained with inorganic compounds were similar to those obtained by NESSLER. In addition, he found that nearly all organic substances promote burning with a flame.

It has been suggested by BARTH (**2**), that the burn and characteristic glow of a good tobacco is due to a reduction of the alkali salts to oxides and small quantities of free metal, and that such a reduction would increase the efficiency of these metals as oxidizing catalysts.

BEHRENS (**4**), as a result of his investigational work on the burning qualities of tobacco, states that a high content of potassium in combination with citric, malic and oxalic acids largely determines the burning quality. GARNER (**9**, p. 19), is also of the opinion that potassium in such combinations is highly desirable, and further suggests that the favorable action of the carbonate obtained from the combustion of these substances may be due to its effect of functioning as a catalyst in combustion by taking up carbon dioxide and forming the acid carbonate at the most favorable moment and losing it later on. KRAYBILL (**12**), however, obtained results which seem to disprove this theory.

RIDGWAY (**22**) found a striking relationship existing between the fire-holding capacity and the degree of aggregation of various salts normally present in tobacco leaves. Under certain conditions of curing, it appears that there is a noticeable tendency for calcium and magnesium to crystallize as malates, citrates and oxalates. As these bases do not appear to promote the fire-holding capacity to any considerable degree, such a process is desirable, as relatively larger quantities of the tissues are thus left free to burn. He found that where such a crystallization did not take place, the burning quality was always poor.

According to LOEW (**13**, p. 38), the more oxidizable material, and the more oxidation going on within the tobacco leaf, the more will the crystals or "grain" develop in the curing and sweating processes and hence it will in many cases, although by no means in all, confirm the idea of some tobacco manufacturers that a well-developed grain is a good sign of the quality of the tobacco.

That chlorine exerts a deleterious effect on the burning quality of cigar-leaf tobacco is quite definitely established. MAYER (**14, 15, 16**), BEHRENS (**4, 5**), NESSLER (**17, 18, 19, 20**), FESCA (**6**), GARNER (**9**, p. 18), AMES and BOLTZ (**1**, p. 191), OLSON (**21**, p. 11), JENKINS (**11**, p. 95), and others have reported on the poor quality of cigar-leaf tobacco fertilized by chlorine-containing material. JENKINS (**10**) also states that while some chlorine is absolutely essential for the development of the tobacco plant, a large excess may prove deleterious to the burn.

Some work has been done to show a relation between the water-, ether-, and alcohol-soluble constituents of the leaf to its burning quality. GARNER (**9**, p. 18), made water extractions and found that the extracted leaf lost its glowing capacity. On examining the extract, he found that it contained the chloride, sulphate, nitrate, malate and citrate of potassium, ammonium and nicotine, and small quantities of lime and magnesium. Extracts from both good-burning and poor-burning leaves contained about the same quantities of potassium but the inferior leaf contained a much higher percentage of mineral acids. Hence he concluded that the potassium salts of organic acids such as malic and citric are the chief factors controlling the burn. Extraction with alcohol had little or no effect on the burn. It has also been shown by GARNER (**9**, p. 8) that some of the weaker organic acids, on the order of tannic, in combination with nicotine, are deleterious to the burning quality.

GRAHAM and CARR (**7**) made petroleum ether, sulphuric ether, alcohol and hot water extractions of tobacco and showed a relation between poor-burning quality and a high extractive content. They showed that tobacco fertilized with sodium nitrate or muriate of potash yielded a high amount of extractives.

Purpose and plan of the experiment

The object of this investigation was to make a study of the chemical composition of a given strain of cigar-leaf tobacco[3] in relation to its burning qualities, particularly as regards its fire-holding capacity. Particular attention was to be given to the determination of the quantity of ether-soluble organic acids present in cured and fermented samples, and also to their form of combination, as measured by the water-soluble and insoluble alkalinity of the ash. In addition, the effects of chemical treatments and climatic conditions were to be given due consideration.

MATERIALS USED

Eighteen plants, carefully selected from each of ten plots, 1/30th of an acre in area, located at Ephrata, Lancaster County, Pennsylvania, were harvested at the end of the growing season in 1925 and removed to the curing shed, where they were air cured. These samples were taken from the shed at the end of the curing season and dried under laboratory conditions. When they were thoroughly dry, the leaves, stems and stalks were separated, finely ground and transferred to air-tight containers and kept for analysis. This same procedure was followed in 1926.

The chemical treatment of the plots from which the samples were taken is shown in table I.

[3] HIBSHMAN strain, Pennsylvania Broadleaf.

TABLE I

FERTILIZER TREATMENTS OF THE EXPERIMENTAL PLOTS

PLOT	TREATMENTS[a]										
	MANURE	SULPHATE OF POTASH	MURIATE OF POTASH	NITRATE OF POTASH	MANURE SALT	BONE FLOUR	PRECIPITATED BONE PHOSPHATE	ACID PHOSPHATE	UREA	AMMONIUM NITRATE	COTTON-SEED MEAL
	tons	lbs.	lbs.	lbs.	lbs.	lbs.	lbs.	lbs.	lbs.	lbs.	lbs.
A–1	10	.....	200	.....	.....	250	.....	.....	.....	.....	400
A–2	10	200	.....	.....	.....	.....	.....	300	.....	.....	500
A–5	10	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
A–6	10	200	.....	.....	.....	.....	.....	300	.....	.....	500
A–9	10	.....	.....	.....	400	250	.....	.....	.....	.....	400
A–10	10	.....	200	.....	.....	.....	.....	300	.....	.....	500
B–1	10	.....	200	.....	.....	.....	.....	300	65	.....	.....
B–2	10	.....	.....	214	.....	.....	120	.....	.....	.....	.....
B–3	10	200	.....	.....	.....	.....	.....	300	65	.....	.....
B–6	10	200	.....	.....	.....	.....	.....	300	.....	85	.....

[a] The potash, phosphoric acid and nitrogen-carrying materials, with the exception of manure and cottonseed meal constituted acre applications of approximately 100 lbs. of potash, 48 lbs. of phosphoric acid and 38 lbs. of nitrogen.

Methods of analysis

In determining the total organic acid content of the different samples of tobacco, 10 grams of the material were transferred to a porcelain evaporating dish and 15 cc. of a 20 per cent. H_2SO_4 solution added. The mixture was then stirred thoroughly by means of a spatula, and finally enough powdered pumice stone was added to make the mass appear fairly dry. The mixture was then placed in a paper thimble and extracted with anhydrous ethyl ether for 24 hours. To the ether extract was added 200 cc. of water, the ether allowed to evaporate, the solution cooled, filtered and made up to a volume of 250 cc. Aliquots of 25 cc. were then taken and titrated with 0.04 N. NaOH, using phenolphthalein as an indicator.

In determining the crude ash and alkalinity of the ash, 2 grams of the sample were transferred to a platinum dish and carefully charred, the residue treated with water and filtered. The insoluble portion was placed again in the dish and ignited to constant weight. The filtrate was next added to the dish, evaporated, and the total residue carefully heated, cooled and weighed. The weight of the residue was calculated as crude ash.

About 150 cc. of hot water were next added to the crude ash and filtered. The residue on the filter-paper was washed well and the filtrate obtained was titrated with N/10 H_2SO_4, using methyl orange or xylene cyanal methyl orange as indicator.

The residue on the filter-paper was then placed in a platinum dish, treated with an excess of N/10 H_2SO_4, and the solution heated to boiling and filtered. The excess acid was then titrated with NaOH standard solution and the insoluble alkalinity was calculated accordingly. The usual methods of analysis were employed to determine the remaining constituents.

Experimental

Experiment I

Samples of the 1925 crop were taken and the more important constituents of the web determined. The results obtained are given in table II.

Experiment II

The total ether-soluble organic acid content of the stems, stalks and web portions of the cured samples, and also of the leaves of the fermented samples were determined. In addition, the total water-soluble and insoluble alkalinity of the web portions of the cured material were likewise determined.

The number of cc. of N. NaOH required to neutralize the ether-soluble organic acids in 100 grams of the air-dry material, and the number

TABLE II

THE PERCENTAGE COMPOSITION OF THE WEB OF CURED TOBACCO LEAVES OF THE 1925 CROP AS INFLUENCED BY FERTILIZER TREATMENT[a]

PLOT	CRUDE ASH	SiO_2	P_2O_5	CaO	MgO	SO_3	K_2O	Cl	N	NICOTINE
	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.
A– 1	24.08	2.10	0.50	7.88	1.25	1.46	1.89	1.12	4.70	4.89
A– 2	23.79	1.88	0.40	7.06	0.96	1.49	1.94	0.12	4.33	5.14
A– 5	23.99	2.52	0.40	7.30	1.24	1.40	1.93	0.19	4.38	4.61
A– 6	22.78	2.23	0.43	7.58	1.32	1.14	1.65	0.17	4.20	5.16
A– 9	24.15	2.14	0.44	7.93	1.21	1.27	1.95	2.29	4.37	4.56
A–10	23.10	2.23	0.44	7.64	1.15	1.35	1.97	0.95	4.63	4.79
B– 1	24.45	3.53	0.45	7.08	1.16	1.29	1.84	1.26	4.73	4.51
B– 2	23.32	2.09	0.48	7.83	1.31	1.61	1.97	0.19	4.60	4.82
B– 3	23.34	2.22	0.42	7.30	1.08	1.45	1.93	0.22	4.67	4.90
B– 6	23.59	3.87	0.43	7.17	1.14	1.28	2.03	0.25	4.87	4.09

[a] All percentages based on moisture-free material.

of cc. of N. H_2SO_4 required to neutralize the alkalinity of the ash of 100 grams of the same material are given in table IV.

EXPERIMENT III

Samples of the 1926 crop were taken after curing and determinations made of crude ash, chlorine, lime, magnesia and potash content of the web portions. In addition, the alkalinity and ether-soluble organic acid con-

TABLE III

THE CHEMICAL COMPOSITION OF THE 1926 CROP OF TOBACCO

PLOT	CRUDE ASH	Cl	K_2O	CaO	MgO	ALKALINITY OF ASH			ETHER-SOLUBLE ORGANIC ACID CONTENT
						Sol.	Insol.	Total	
	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.	cc.	cc.	cc.	cc.
A– 1	18.03	0.94	2.13	6.39	0.94	36	293	329	305
A– 2	22.85	0.17	2.29	6.43	0.89	48	283	331	281
A– 5	23.79	0.24	2.55	6.44	0.75	34	278	312	290
A– 6	21.08	0.17	2.38	6.29	0.86	51	323	374	290
A– 9	23.34	1.95	2.69	6.56	0.82	17	333	350	274
A–10	22.80	0.95	2.33	6.79	1.06	37	326	363	293
B– 1	22.78	1.07	2.04	6.83	1.09	25	296	321	286
B– 2	22.92	0.21	2.13	6.79	0.95	40	320	360	293
B– 3	22.54	0.19	3.37	6.75	0.88	39	306	345	283
B– 6	20.70	0.21	2.19	6.78	1.40	32	320	352	300

tent were also determined on the same samples. The results are presented in table III.

TABLE IV

THE TOTAL ETHER-SOLUBLE ORGANIC ACID CONTENT AND ALKALINITY OF THE 1925 CROP OF TOBACCO

SAMPLE	PLANT PART	CURED MATERIAL				FERMENTED MATERIAL
		ALKALINITY OF ASH			ETHER-SOLUBLE ORGANIC ACID CONTENT	ETHER-SOLUBLE ORGANIC ACID CONTENT
		Sol.	Insol.	Total		
		cc.	cc.	cc.	cc.	cc.
A– 1	Web	11	343	354	333	332
A– 1	Stem				234	
A– 1	Stalk				100	
A– 2	Web	39	322	361	336	
A– 2	Stem				300	
A– 2	Stalk				107	
A– 5	Web	22	332	354	347	351
A– 5	Stem				327	
A– 5	Stalk				139	
A– 6	Web	13	343	356	351	345
A– 6	Stem				304	345
A– 6	Stalk				128	
A– 9	Web	8	315	323	314	325
A– 9	Stem				167	
A– 9	Stalk				95	
A–10	Web	13	232	336	331	316
A–10	Stem				223	
A–10	Stalk				106	
B– 1	Web	14	317	331	313	305
B– 1	Stem				225	
B– 1	Stalk				103	
B– 2	Web	19	353	372	362	341
B– 2	Stem				311	
B– 2	Stalk				118	
B– 3	Web	35	309	344	345	324
B– 3	Stem				319	
B– 3	Stalk				163	
B– 6	Web	19	321	340	333	317
B– 6	Stem				324	
B– 6	Stalk				137	

Experiment IV

Samples of the web portions of the cured leaves of the 1925 and 1926 crops were taken and their hydrogen-ion concentration determined by the quinhydrone method (3). The results obtained are given in table V.

TABLE V

The hyrrogen-ion concentration of the 1925 and 1926 crops of tobacco

Year	Plots									
	A–1	A–2	A–5	A–6	A–9	A–10	B–1	B–2	B–3	B–6
	pH	pH	pH	pH	pH	pH	pH	pH	pH	pH
1925	5.96	5.96	5.78	5.76	5.97	6.01	5.38	5.74	5.34	5.61
1926	5.95	6.19	6.08	6.16	6.10	6.20	6.37	6.78	6.08	6.27

Experiment V

Burning tests were conducted on strips of the fermented leaves of the 1925 and 1926 crops of tobacco. These strip tests of the fermented tobacco are shown in table VI.

TABLE VI

Strip tests[a] of the 1925 and 1926 crops of tobacco after fermentation

Plot	Burn 1925	Burn 1926
A– 1	Poor	Poor
A– 2	Fair	Fair to good
A– 5	Poor	Poor
A– 6	Good	Good to fine
A– 9	Poor	Poor
A–10	Poor	Poor
B– 1	Poor	Poor
B– 2	Good	Good to fine
B– 3	Fair to good	Good to fine
B– 6	Fair	Fair to good

[a] Definitions of terms in strip tests:

Very poor = tobacco does not hold fire. Coals badly.

Poor = tobacco holds fire but a few seconds. Coals badly.

Fair = tobacco holds fire over five seconds. Coals moderately.

Good = tobacco holds fire evenly over ten seconds. Coals slightly.

Fine = tobacco holds fire evenly until consumed. Coals very slightly.

Excellent = tobacco burns evenly until consumed without coaling and with light colored ash.

Weather conditions

Certain observations were made as to the weather conditions which prevailed during the growing seasons of 1925 and 1926. In 1925 the temperature was relatively high from May 31st until June 10th, and no precipitation occurred until June 25th. July was marked by severe electrical storms and heavy washing rains. The temperature rose gradually during the first two weeks of the month of August. No rain occurred from August 15th to September 13th. The plants were harvested shortly after this period.

The weather conditions for 1926 were quite different. It was moist and cool until June 10th, after which time the temperature rose to a relatively high degree and severe storms occurred during this hot period. The hot weather continued through July and August, but rain storms were quite frequent, breaking all previous rain records for August, and the excessive heat lasted until the plants were harvested. Following are the figures showing the actual precipitation during the growing season of 1926.

Month	Rainfall inches
June	4.31
July	4.57
August	9.12
September	4.47

No accurate measurement of the rainfall during the growing period was made in 1925, but, on the whole, the precipitation was much less than for the season of 1926.

Discussion of results

Experiment I, table II, shows that the chemical composition of the 1925 crop, from an elemental standpoint, was rather constant regardless of the fertilizer treatment. More chlorine, however, was found in the plants receiving chlorine-bearing compounds. The water-soluble and insoluble alkalinity of the ash also varied to a certain extent as shown by experiment II, table IV. The form of potash used appeared to play a part in causing this variation. Where the plants received muriate of potash, the water-soluble alkalinity was less as a rule than where the sulphate was used. A higher total alkalinity was obtained where potassium nitrate was applied. This table also shows a very wide potassium-calcium ratio, a wider ratio than is commonly found in good burning tobacco.

Analyses of the crop of 1926, according to experiment III, table III, show that approximately the same quantity of chlorine was present as in the 1925 crop. On the other hand, the lime and magnesia contents were lower and the potassium content was higher in the 1926 samples, but the potassium-calcium ratio was still too wide for satisfactory combustion. Where chlorine was present in relatively large quantities, a low water-soluble alkalinity of the ash was again apparent. On the whole, however, the water-soluble alkalinity of the 1926 crop was higher than that of the 1925 crop. The principal results obtained from these investigations show, with few exceptions, that there is a correlation between the water-soluble alkalinity of the samples and their burning qualities, and that this effect is more pronounced than that of the relative quantity of ether-soluble organic acids. It appears evident also that the form and amount of potash, and to a certain extent the season, have a great deal to do with the total quantity of water-soluble alkalinity present. If this is true, it is important to account for these effects.

Owing to the limited number of samples studied, it would not be proper to draw many definite conclusions, but it appears that these samples contain too little potassium and too great a quantity of calcium for the most desirable combustion. The data also indicate that the sulphate of potash is more desirable as a carrier of potassium than is the muriate. If potassium functions normally within the plant, it must act as a base. Combined with chlorine or sulphur it does not have the desirable property of neutralizing organic acids to any considerable extent. As chlorine is not normally required in any large quantities by the tobacco plant, it would probably tend to remain in combination with potassium, at least to a certain extent. On the other hand, sulphur may be utilized in relatively large quantities, and under these conditions more potassium would be rendered available for the neutralization of organic acids. As a matter of fact, however, analyses of the plants showed that the sulphur content was not increased by the addition of sulphates, so the reason for the difference in the basic properties of potassium from the sulphate, as compared with the muriate, must be sought elsewhere.

It is a well-known fact that the principal replaceable base of soil colloids, in a calcareous district such as Lancaster County, Pennsylvania, is calcium, and that the addition of potash salts to such a soil should lead to the replacement of calcium from such combinations and the formation of calcium salts. At the same time, it is possible to reverse this process. In other words, there is a competition existing between calcium and potassium, whereby either one or the other goes into colloidal combination. Under laboratory conditions, a sample of soil may be treated with muriate of pot-

ash and the replaced calcium may be completely removed by leaching, leaving potassium behind in basic form. On the other hand, it is quite possible to remove all absorbed potassium by the use of a solution of calcium chloride, leaving calcium behind in colloid combination and in basic form. Under natural conditions, neither of the above effects is ever observed, but a relatively dry year, such as in 1925, would have a different effect on the availability of potassium than would a comparatively wet year, as in 1926. Continual leaching of the soil would have the effect of rendering potassium available for absorption in basic form by the tobacco plants, while such a condition would not hold true under relatively dry conditions where soluble calcium salts tend to accumulate in the upper soil layers. Under the former conditions the tendency would be, in the light of our present knowledge, for potassium to assume the carbonate form. Under the latter condition, potassium would tend to assume the form of the muriate or sulphate, as the case may be. These reasons would tend to explain, in a measure, the beneficial effects of a wet season on the burning qualities of Lancaster County cigar-leaf tobacco, but would not necessarily hold true to any appreciable extent on soils lacking in colloidal material, unless the presence of relatively large quantities of calcium tends to inhibit the maximum absorption of potassium by plants.

The beneficial effect of sulphate of potash in a dry year, as compared with the muriate, may be due in part to the difference in chemical activity of the calcium compounds formed where these materials are added, the sulphate being more insoluble and less active chemically than the chloride, and not having the power of replacing potassium, which is possessed by the latter under ordinary conditions.

On the whole, the hydrogen-ion concentration of the 1926 crop was more nearly neutral than the 1925 crop. This would tend to have a desirable effect on the burn in an indirect manner, as it would more nearly approximate the optimum conditions for oxidase activity in the curing and fermentation processes.

Summary and conclusions

This investigation had for its purpose the study of the effect of certain constituents of the leaf in relation to the burning quality of cigar-leaf tobacco. As a result of these studies the following conclusions seem justified:

1. The ether-soluble organic acids of the plant appear to occur almost wholly in combination with the alkali and alkali earth metals, as measured by the water-soluble alkalinity and insoluble alkalinity of the ash respectively.
2. In practically all cases there is a parallelism between the burning qualities and the water-soluble alkalinity of the ash.

3. The season and the form of potash supplied as a fertilizer affected the water-soluble alkalinity of the ash in practically all cases.
4. The phenomenon of base exchange in the soil and the removal of certain active substances through leaching probably have considerable to do with the burn and composition of tobacco, but the difficulty involved in the absorption of potassium, under different conditions, in highly colloidal soils such as those of the experimental plots, should also be given consideration.
5. The lime content of the samples used in this experiment was too high and the potash content too low for maximum burning quality. Further work is in progress relative to increasing the potash content and lowering the calcium content of the tobacco grown in Lancaster County, Pennsylvania.

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MUCK-SOIL REACTION AS RELATED TO THE GROWTH OF CERTAIN LEAF VEGETABLES[1]

E. V. HARDENBURG

(WITH TWO FIGURES)

Soil reaction studies as related to the growth and character of crop plants have engaged the thought of numerous research workers during the past fifteen years. In fact, the reports of the majority of these studies bear dates more recent than the pioneer studies of HARTWELL and DAMON (3) which resulted in the classification of many economic plants according to their tolerance of soil acidity or their relative response to applications of lime. A review of the literature indicates that very few such studies with vegetables have been made and most of these have dealt with vegetables grown on mineral soils.

Review of literature

ARRHENIUS (1) determined the optimum pH for several crops, among which were sugar beets pH 7.5, radishes and rutabagas pH 7.0, peas pH 6, and turnips pH 4. He decided that most plants have two optimal points rather than a simple gradient. His method was colorimetric and may be criticized as giving, at best, only approximate results which obviously do not agree with more recent experiments. CRIST (2) grew Grand Rapids lettuce in nutrient water cultures of varying hydrogen-ion concentration and obtained maximum green weight at pH 5.0, with decreasing yields as the alkalinity increased from this point. Similarly, he grew lettuce in a soil culture of two-thirds acid muck and one-third coarse sand well fertilized with a 0-8-10 mixture and treated with hydrated lime in varying amounts. With each increase in lime application the green weight of lettuce and the ratio of top to root decreased. CRIST ascribed the injurious influence of the lime to change in permeability of the plant membrane, thus lowering its absorption of both nutrients and water, thereby causing limited malnutrition. LIPMAN, MCLEAN, and LINT (5), using sulphur in varying amounts on Sassafras sandy loam in a greenhouse experiment, obtained decreasing yields of barley, buckwheat and mustard, while radish yields were increased by the same treatments. MARTIN (7) applied sulphur flour in 300 pound per acre increments up to 1,200 pounds, to 1/60 to 1/40 acre

[1] Problem pursued in the Office of Horticulture, U. S. Department of Agriculture. Acknowledgment is made to Mr. J. H. BEATTIE, at whose suggestion and under whose advice the problem was undertaken, and to Dr. E. T. WHERRY, of the Bureau of Chemistry and Soils, for advice and assistance with the chemical phases.

plots of potatoes on three soil types and determined the hydrogen-ion concentration of the soil solution at frequent intervals from May 19th to August 16th. The acidity in all treatments increased up to June 24th, after which there was a gradual decrease. MARTIN explained this decrease in acidity by suggesting that all of the sulphur may have been oxidized by that time (71 days after application). Whereas the sulphur treatments decreased the pH value from 6.27 for the check plot to 5.10 for the plot receiving 1,200 pounds of uninoculated sulphur, the highest total yield was obtained from the last named plot. This would indicate an optimum pH value for potatoes under these conditions as low as 5.0. THERON (**8**) grew alfalfa, cotton, cucumbers, barley, and Bermuda grass in nutrient water cultures in which a wide range in pH values was set up and maintained by the addition of sulphuric acid and sodium hydroxide. Judging by the effects on both yield of green weight and appearance of the plants, he classified these crops according to their sensitivity to both H-ion and OH-ion concentration. Alfalfa and cucumber, showing a range of pH 4.8 to pH 6.0 for optimum growth and appearance, were classed as very sensitive, while barley and Bermuda grass having an optimum pH range of 4.5 to 7.0 and 4.5 to 8.0 were classed, respectively, as resistant and highly resistant.

ZIMMERLEY (**10**) has contributed what is perhaps the most detailed report on the relation of soil reaction to the growth of a given vegetable. He grew spinach, in a three-year experiment, in 15-inch glazed tile cylinders filled to a depth of 24 inches with each of three highly acid soils used in spinach production in the Norfolk area. Hydrated lime in amounts ranging from 1000 to 10,000 pounds to the acre was used to vary the pH values from 4.6 to 7.5. Yields of spinach increased in direct ratio to the amount of lime added up to pH 7.0 to 7.5 in 1924 and up to 6.5 to 6.9 in 1925 and 1926. Field test plots essentially substantiated the cylinder results by showing an optimum range of pH 6.5 to 7.0, depending on soil type and season. On these very acid soils, each 1000 pound increment of hydrated lime appreciably lowered the hydrogen-ion concentration up to the point of neutrality. Beyond pH 7.0, the increase in alkalinity was only pH 0.1 for each 1,000 pound application. In other unpublished work, ZIMMERLEY reports the optimum pH for lettuce grown on sandy soil as 5.6, growth at pH 4.7 being extremely poor. With beets, results were poor when the pH value went above 6.0. He furthermore reports danger of chlorosis in spinach with pH values greater than 7.0, instances of chlorosis being observed at pH values as low as 6.5.

The problem

Muck-land production of vegetables is not only increasing rapidly but there is an increasing interest in the number of kinds of vegetables which

may be adapted to soils of organic origin. Our markets are already well supplied with the more commonly produced muck crops from California, the middle west and localized areas in the east. The problem is now largely one of determining under what conditions other crops as well as these can be most efficiently produced. The subject of muck-soil reaction is by no means new but certainly too little is known of its basic character to permit of much confidence in our recommended cropping systems. The majority of our peat and muck soils now under cultivation are at least slightly acid, although many of them are classed as high in lime. So-called high-lime muck soils are obviously, not necessarily alkaline in reaction. A review of the literature indicates that many, perhaps most, of our common vegetables produce optimum yields on soils the pH value of which ranges between 5.0 and 6.5. There is great need for soil reaction studies of muck and peat soils with reference both to the influential factors and the response of the plant affected.

The primary purpose of the problem reported here was to study the performance of certain leafy vegetables when grown on a standard well-fertilized muck soil of varying hydrogen-ion concentration. Of secondary importance to the problem were such factors as comparative toxicity to these plants of active aluminum and sulphur under the conditions of the experiment, and absorption of aluminum, iron and calcium as determined by ash analysis of the oven-dried material from each culture treatment.

Materials and method

Lettuce, mustard, endive, and parsley were selected for study first, because they represent different degrees in sensitivity to soil acidity in the order named, as determined at the Rhode Island Station (**3**) and second because, being leafy vegetables, the yields could readily be compared in terms of oven-dry weights.

The soil used in all cultures was well oxidized muck from the Kankakee marsh area near South Bend, Indiana, the initial hydrogen-ion concentration several days after the fertilizer and manurial treatment being pH 5.0. The original soil as used in all checks and as maintained, except for the soil reaction treatments, analyzed as follows:

Moisture	Per cent. 53.47	
	Air-dry basis	Oven-dry basis
Ash	2.08	4.35
Phosphoric oxide, P_2O_5	0.18	0.38
Nitrogen, N	1.22	2.62
Lime, CaO	1.77	3.81
Potash, K_2O	0.13	0.28

These data indicate that the soil would class as low-lime muck having a comparatively narrow nitrogen-carbon ratio and, according to WHERRY (9), an active acidity of about 100 units (mediacid). In setting up the cultures, 10-inch clay flower pots were each filled with 15 pounds of thoroughly mixed and fertilized soil, the fertilizer treatment being 2000 pounds of a 2-8-10 mixture and 40 tons of clear cow-manure per acre. The fertilizer treatment was computed on the basis of 1,000,000 pounds of surface soil per acre and thoroughly mixed with the soil before potting. A uniform moisture condition was maintained by watering each culture to original weight twice a week throughout the growth period. After several preliminary trials with the soil reaction treatments to establish a fairly wide range in pH values, the soil was finally potted and the treatments applied on January 5th. The plant material used, together with dates of seeding, pricking-out, potting and harvesting are given in table I.

TABLE I

MATERIALS AND TREATMENT CALENDAR

CROP	VARIETY	SOURCE	SEEDED IN FLATS	PRICKED OUT	POTTED	HARVESTED
Lettuce	Grand Rapids	Bolgiano	11/22	12/6	1/10	3/29
Mustard	Giant Curled	Stokes	12/15	12/21	1/10	3/18
Endive	Green Curled	Stokes	11/22	12/21	1/10	4/6
Parsley	Giant Moss Curled	Stokes	11/2	11/22	1/10	4/15

Sufficient plants were grown to permit selection for size and uniformity. In general, the second pair of true leaves was developed at time of potting. All plants grew normally except no. 7 in the endive series (2000 pounds of sulphur) this remaining stunted and refusing to grow normally, although at no time was there evidence of disease or lack of chlorophyll development. A total of 32 plants of each kind was potted, 4 for each of the 8 soil reaction treatments. The original plan called for the use of aluminum sulphate to provide the higher hydrogen-ion concentrations. Preliminary tests indicated that even large applications would not result in pH greater than 5.0. Furthermore, some apprehension was felt that this material might result in injury from aluminum toxicity. Accordingly, commercial sulphur flour (uninoculated) was also used to provide the extremes of acidity in the series. Chemically pure calcium carbonate was used in varying amounts to provide the lower hydrogen-ion concentrations. Single plants were grown in each culture. Four untreated cultures were grown in each series to provide checks. This made a total of 128 cultures. All soil reaction treatments

were applied to the surface and thoroughly mixed with the upper 2 inches of soil, the aluminum sulphate being first dissolved in distilled water. These treatments consisted of 4000 and 2000 pounds of sulphur; 8000 and 2000 pounds of aluminum sulphate; check; 4000, 12,000, and 20,000 pounds of chemically pure calcium carbonate per acre, providing a range in pH values from approximately 4.0 to 7.0, respectively. The double wedge color comparator apparatus adapted and recommended by Dr. E. T. WHERRY, of the U. S. Bureau of Chemistry for soil extracts, was used in making the hydrogen-ion determinations. Readings were made on about one-half the cultures, including some from each series, at intervals of about 2 weeks throughout the growth period. The pH values given in the summary tables represent the average of these readings for each treatment. Before drawing the sample for each determination, a portion of the surface soil to an average depth of about 1 inch was mixed and the necessary quantity taken from this area.

TABLE II

RELATION OF HYDROGEN-ION CONCENTRATION TO GREEN WEIGHT OF TOPS IN LETTUCE, MUSTARD, ENDIVE, AND PARSLEY. (AVERAGE OF 4 PLANTS AND 5 TO 12 DETERMINTIONS FOR EACH TREATMENT)

TREATMENT	RANGE	LETTUCE	MUSTARD	ENDIVE	PARSLEY
Per acre	pH	gm. per plant	gm. per plant	gm. per plant	gm. per plant
4000 lbs. sulphur	3.95–4.05	375.00	213.43	344.13	111.95
2000 " sulphur	4.55–4.62	332.88	301.53	384.57	129.75
8000 " $Al_2(SO_4)_3$	4.68–4.76	410.88	318.38	366.80	111.75
2000 " $Al_2(SO_4)_3$	4.88–4.97	428.13	275.28	378.30	128.85
Check	5.00–5.04	423.75	274.63	473.75	145.50
2 tons $CaCO_3$	6.01–6.56	428.63	302.18	429.25	149.88
6 " $CaCO_3$	6.77–6.88	397.88	301.75	438.25	158.98
10 " $CaCO_3$	6.92–7.00	347.13	300.48	452.78	152.33

Reference to table II shows that even 10 ton applications of calcium carbonate failed to provide an alkaline soil reaction. Of the 16 cultures treated with the 10 ton application, there were several pH readings which exceeded 7.0 but in no case was the value greater than pH 7.1. The exceedingly high buffer effect here evident may be attributed to the high organic content and the assumed high colloidal complex common to this and similar muck soils. In this connection it should be noted that whereas the 2 ton lime application raised the pH value from approximately 5.0 to 6.5 or decreased the active acidity by nearly 100 units, increased amounts of lime affected the acidity very much less per unit application. The range in pH values shown in table II also indicate that the active acidity was much more affected by the

sulphur than by the aluminum sulphate per unit of application. The aluminum sulphate treatments not only did not result in apparent toxicity to any of the cultures but also were comparatively inefficient in increasing acidity of the soil solution.

The optimum pH range indicated for all four vegetables was obviously between 4.5 and 7.0. For lettuce the optimum range appears to be between pH 5.0 and 6.5. A typical series of Grand Rapids lettuce plants grown under the conditions of table II is shown in fig. 1. This may be considered

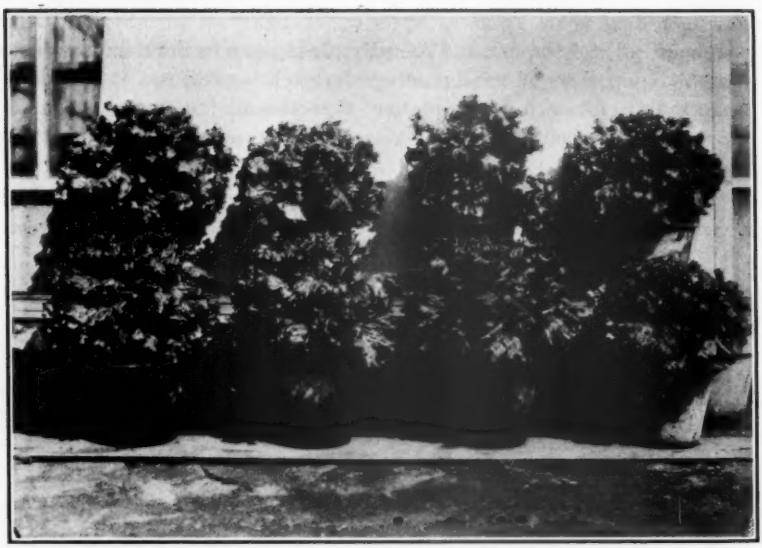

FIG. 1. Grand Rapids lettuce: A typical series from the muck soil treatments. Left to right: upper row, 4000 lbs. sulphur; 2000 lbs. sulphur; 8000 lbs. aluminum sulphate; 2000 lbs. aluminum sulphate. Lower row, check; 2 tons lime; 6 tons lime; 10 tons lime.

in fairly close agreement with the data of CRIST (**2**) whose optimum for this variety of lettuce was pH 5.0. In further agreement with CRIST are the data showing decreased green weight yields for the heavier lime applications. The lettuce yield was approximately as high at pH 4.0 to 4.5 as at pH 7.0. Further study of table II shows most consistent response to increasing alkalinity by the parsley, second endive, with results for mustard and lettuce favored by a reaction classing as mediacid to minimacid (WHERRY, **9**). A typical series of the moss curled parsley is shown in fig. 2.

These results cannot be construed as confirming the classification of these vegetables as determined at Rhode Island (3) where, however, the soil used was a greenhouse potting soil of mineral origin.

FIG. 2. Moss Curled parsley: A typical series from the muck soil treatments. Left to right: upper row, 4000 lbs. sulphur; 2000 lbs. sulphur; 8000 lbs. aluminum sulphate; 2000 lbs. aluminum sulphate. Lower row, check; 2 tons lime; 6 tons lime; 10 tons lime.

Ash analysis of lettuce

Other workers in this field have made some study of the relation of soil reaction to ash content and ash analysis with particular reference to the absorption of calcium and aluminum under various treatments, CRIST (2), LINE (4), MAGISTAD (6), and perhaps many others not referred to in this discussion. Inasmuch as both lime and aluminum sulphate were used in this experiment, it was thought advisable to study their effect on the ash content of one of the crops grown. Lettuce was chosen because of its importance as a truck crop. The ash content of the oven-dried lettuce tops, together with the percentage composition and actual weight per plant of Al_2O_3, Fe_2O_3 and CaO are given in table III.

Obviously, there was no consistent relationship between hydrogen-ion concentration and either percentage of total ash or of aluminum-, iron-, and calcium-oxide absorbed. It is of some interest that even less aluminum was

TABLE III

ASH ANALYSIS[a] OF OVEN-DRY LETTUCE TOPS UNDER VARIOUS MUCK-SOIL TREATMENTS

TREATMENT	REACTION VALUE	ASH IN DRY MATTER		Al_2O_3		Fe_2O_3		CaO	
Per acre	pH	Per cent.	gm. per plant	Per cent.	gm. per plant	Per cent.	gm. per plant	Per cent.	gm. per plant
4000 lbs. sulphur	4.03	24.06	6.98	0.321	0.022	0.055	0.0038	2.27	0.158
2000 " sulphur	4.55	21.72	5.74	0.433	0.025	0.030	0.0017	2.06	0.118
8000 " $Al_2(SO_4)_3$	4.68	23.57	7.25	0.321	0.023	lost	lost	2.16	0.157
2000 " $Al_2(SO_4)_3$	4.92	24.36	7.81	0.413	0.032	0.050	0.0039	2.19	0.171
Check	5.00	22.80	8.03	0.280	0.022	0.035	0.0028	2.21	0.178
2 tons $CaCO_3$	6.38	27.75	9.76	0.334	0.033	0.080	0.0078	2.10	0.205
6 " $CaCO_3$	6.78	22.45	7.55	0.257	0.019	0.040	0.0030	2.08	0.157
10 " $CaCO_3$	7.00	21.94	6.48	0.284	0.018	0.037	0.0024	1.96	0.127
Average	.....	23.58	7.45	0.330	0.024	0.047	0.0036	2.13	0.159

[a] Analyses by RUTH G. CAPEN, analyst, Crop Chemistry Laboratory, U. S. Bureau of Chemistry and Soils.

absorbed under the 8000-pound than under the 2000-pound per acre application of aluminum sulphate. The same is true of sulphur absorption from the two sulphur treatments.

Lack of any toxic effect of the aluminum sulphate treatments on any of the cultures can be explained on either of the theories set forth by LINE (**4**) or MAGISTAD (**6**). LINE'S experiments indicated that aluminum will not remain soluble in soils less acid than pH 4.5, or than pH 3.5 if soluble phosphate is present. On the contrary, it will precipitate out as insoluble aluminum phosphate at pH concentrations higher than those indicated. MAGISTAD'S work indicated that aluminum solubility does not increase rapidly until the acidity has reached at least pH 4.5.

With respect to the lime applications, there is at least some indication in table III that the absorption of aluminum, iron, and calcium decreased with each increment of lime added. These results accord with those obtained by CRIST (**2**) with Grand Rapids lettuce grown under similar conditions so far as aluminum and calcium are concerned. CRIST explained this differential absorption on the thoery that the "effect of applications of lime presumably on the permeability of the tissues was to reduce the intake of every mineral element for which the tissues were analyzed and the heavier the applications, the greater was the reduction, particularly as regards calcium and phosphorus." Unfortunately, the ash was in this case not analyzed for phosphorus. The final absolute water content of these plants decreased as the lime was increased, while the percentage content of water was approximately 92 for all three lime treatments. Here again the data are almost identical with those of CRIST.

Root and top ratios

Both tops and roots of all cultures were oven-dried to constant weight for oven-dry weights. These coincide very consistently with the green weights and, therefore, are not tabulated here. The roots were removed from the soil without washing. About two days intervened after the removal of the tops to allow the soil to dry further and the roots to shake out readily after inverting the pots. Considerable care was used to retrieve the more fibrous branch roots. Probably the best expression of root growth response to the various soil-reaction treatments is as per cent. of top weights. These data are given in table IV.

From the standpoint of average green weight of roots per culture treatment as related to soil reaction, the discussion given for top growth applies equally well. However, no very obvious correlation between hydrogen-ion concentration and root-and-top ratio appears in table IV, except for endive. It is evident here that root development in endive is consistently favored

TABLE IV

RELATION OF HYDROGEN-ION CONCENTRATION TO RELATIVE ROOT AND TOP GROWTH IN LETTUCE, MUSTARD, ENDIVE, AND PARSLEY. (AVERAGE OF 4 PLANTS AND 5 TO 12 pH DETERMINATIONS FOR EACH TREATMENT). (GREEN WEIGHT BASIS.)

TREATMENT	REACTION RANGE	LETTUCE	MUSTARD	ENDIVE	PARSLEY
		ROOTS AS PERCENTAGE OF TOP WEIGHT	ROOTS AS PERCENTAGE OF TOP WEIGHT	ROOTS AS PERCENTAGE OF TOP WEIGHT	ROOTS AS PERCENTAGE OF TOP WEIGHT
Per acre	pH	Per cent.	Per cent.	Per cent.	Per cent.
4000 lbs. sulphur	3.95–4.05	10.26	11.57	13.84	48.28
2000 " sulphur	4.55–4.62	9.91	15.55	14.99	47.77
8000 " $Al_2(SO_4)_3$	4.68–4.76	9.50	15.27	15.29	59.06
2000 " $Al_2(SO_4)_3$	4.88–4.97	11.45	12.28	16.96	44.16
Check	5.00–5.04	9.99	20.12	15.94	46.29
2 tons $CaCO_3$	6.01–6.56	12.72	15.50	15.90	51.01
6 " $CaCO_3$	6.77–6.88	10.41	12.97	16.81	41.82
10 " $CaCO_3$	6.92–7.00	9.06	14.32	19.74	44.20

as the soil-reaction approaches neutrality. The optimum reaction for root development in lettuce was pH 6.38, for mustard pH 5.04, and for parsley pH 4.75. Whereas CRIST (**2**) obtained a consistent increase in proportionate root development in lettuce with increased lime applications, somewhat opposite results are evident in table IV.

Water requirement

Record was kept of all water applied to the cultures throughout the growth period. It has been possible, therefore, to calculate the water requirement as affected by the various treatments. Since the harvest date, and therefore the length of growing season, for each vegetable varied by several days or weeks, no attempt was made to determine the absolute water requirement of each vegetable for purpose of comparison. Inasmuch as glazed pots were not used and no attempt made to prevent evaporation either from the surface of the soil or pots, the data on water requirement given in table V are calculated on the evaporation-transpiration basis.

Although there is no obvious relation between soil reaction and water requirement it may be noted that the maximum requirement for all four vegetables was reached in the more highly acid cultures. Of special interest is the fact that with lettuce, mustard, and parsley the water requirement increased markedly with each increase in lime application. The trend was opposite and less marked with endive. So far as the lime treated cultures

are concerned, these data coincide rather closely with the yields of dry matter and may be thus explained for all four vegetables.

TABLE V

RELATION OF SOIL TREATMENT TO WATER REQUIREMENT. (POUNDS OF WATER PER POUND OF DRY MATTER)

TREATMENT	LETTUCE	MUSTARD	ENDIVE	PARSLEY
Per acre	lbs.	lbs.	lbs.	lbs.
4000 lbs. sulphur	305.0	411.3	372.7	569.1
2000 " sulphur	352.6	283.0	478.3	630.7
8000 " $Al_2(SO_4)_3$	277.5	299.8	392.3	649.0
2000 " $Al_2(SO_4)_3$	286.3	329.7	345.5	568.3
Check	293.7	335.7	380.7	588.4
2 tons $CaCO_3$	261.1	252.0	342.0	512.7
6 " $CaCO_3$	285.6	273.1	338.2	527.6
10 " $CaCO_3$	348.3	283.1	328.6	538.6

Conclusions

The four leaf-vegetables selected for this experiment, because of their previous classification according to sensitivity to soil acidity, were grown in pot cultures on a highly fertilized low-lime muck soil having an initial pH value of 5.0. Sulphur, aluminum sulphate and calcium carbonate were applied in varying amounts to provide a soil reaction ranging from pH 4.0 to pH 7.0. Four cultures each of lettuce, mustard, endive, and parsley were given the eight soil treatments including the check. Due to the highly buffered nature of the muck, these treatments failed to provide as wide a range of hydrogen-ion concentration as had been hoped for. Even the 10 ton lime applications did not give a pH value greater than 7.0. Sulphur proved more active than aluminum sulphate as an acidifying agent. Contrary to expectation, no trace of aluminum toxicity was evident in any of the four vegetables from the 8000-pound application of aluminum sulphate. This is explained on the basis that the buffer influence of the muck soil prevented the development of a sufficiently high acidity to render the aluminum soluble in toxic quantity.

Best top weights of lettuce were obtained within a pH range of 5.0 to 6.5. The increased applications of lime to lettuce reduced the growth of both top and roots, decreased the total ash content, and the absorption of aluminum, iron, and calcium to a moderate extent. This influence is explained on the basis of change in permeability of the root tissues, thereby reducing the intake of the elements mentioned and probably of other

nutrients. Parsley and endive appear to respond to decreasing acidity more consistently than either lettuce or mustard.

No obvious correlation between hydrogen-ion concentration and root-and-top ratio exists for lettuce, mustard and parsley. Root development in endive appears to be favored by a culture medium which approaches neutrality.

The water requirement of lettuce, mustard, and parsley increased rather markedly with each increase in lime application. There was a corresponding, though much less marked decrease in the water requirement of endive for each increment of lime. With all four vegetables, the maximum water requirement was reached in the more acid cultures.

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THE EXTRACTION AND SEPARATION OF CHLOROPHYLL ($\alpha + \beta$) CAROTIN AND XANTHOPHYLL IN FRESH GREEN LEAVES, PRELIMINARY TO THEIR QUANTITATIVE DETERMINATION*

F. M. SCHERTZ

Introduction

This paper is published because of numerous requests which have been made of the author for information regarding the quantitative determination of the four chloroplast pigments in fresh green leaves. The method as described here is a modification of WILLSTÄTTER and STOLL's method and has been developed and used in the Laboratory of Soil-Fertility Investigations, in the course of a series of investigations on the effect of nitrate, potash and phosphate on plant growth and production. It is hoped that the method as modified will prove highly valuable to those doing research in plant physiology as well as to those doing work along more practical lines. It is to be regretted that a satisfactory method for determining the two chlorophyll components separately is not available at the present time. Consequently, workers will have to be contented to know only the total amount of chlorophyll until more satisfactory methods than the ones described by WILLSTÄTTER and STOLL (5), are developed.

The method

The chief feature of the quantitative estimation of the pigments in fresh leaves is the thorough disintegration of the tissue and subsequent extraction with acetone containing only a low percentage of water.

The leaves may first be treated with aqueous acetone (30 per cent.) which removes yellowish plant acids, inhibits enzyme action, and at the same time removes none of the leaf pigments. The aqueous acetone extract is yellowish in color and appears to contain some of the yellow pigments,[1] but ethereal extractions and observations with the spectroscope revealed none of the yellow pigments in the 30 per cent. acetone extracts. This step is usually omitted by the author.

The details of the method are as follows: In a mortar, 25 cm. in diameter, are put 10 grams of fresh finely chopped or cut leaves[2] and a

* Soil-Fertility Investigations, U. S. Department of Agriculture, Washington, D. C.

[1] The water soluble yellow pigments present here are flavones.

[2] The leaves should contain no petioles nor stems and should be representative of the plants studied. Practice has shown that if the leaves are dried, the pigments decompose more or less and consequently drying is never recommended.

little sodium carbonate to neutralize the acidity. The leaves are quickly mashed (5–30 min.) and ground thoroughly with 25 grams or more of washed quartz sand which serves to disintegrate the leaf substance. The leaves are first ground finely without the addition of any solvent. It is absolutely necessary here to grind till all of the tissue is thoroughly disintegrated if one expects the extraction to be quantitative. There are now added 50–100 cc. of pure acetone, and the material is ground again thoroughly, after which the whole is filtered through a filter-paper on a Buchner funnel 10 cm. in diameter. The leaf substance is then washed with pure acetone until the filtrate runs off colorless. Finally, wash by pouring 100 cc. or more of ether on the filter. The combined filtrate will be colored green and will contain carotin, xanthophyll, and both of the chlorophylls; in addition it contains as impurities, flavones and anthocyanins when they are present in the plant tissue which is being extracted. Complete extraction will require from 100–250 cc. of acetone and 75–150 cc. of ether. Add 50 cc. more of ether if necessary, for the larger the volume of ether, the easier it will be to wash out the acetone.

After the combined ether-acetone extract is obtained it is poured into a liter separatory funnel, and the acetone is then washed out by means of distilled water. First, wash by pouring the water through the ethereal acetone solution, or, better, by pouring the distilled water through a funnel. Add a small amount of water at first in the above manner, till a colorless or only slightly tinted yellow aqueous layer separates and then run off the aqueous layer. The yellow in the aqueous layer consists largely of flavones which may be tested for by adding alkali which causes the yellow color to deepen. Repeat this several times and then wash by gently rotating the liquids. If emulsions form they may be broken by washing with water containing sodium chloride.

In order to remove the remainder of the flavones, wash with a one per cent. solution of sodium carbonate. The flavones will come out as a deep yellow solution. If anthocyanins are present they also will be separated from the ethereal layer of carotin, xanthophyll and the chlorophylls, and will come out in the sodium carbonate wash water. The flavones and anthocyanins will be separated practically quantitatively while the chlorophylls and yellow pigments will remain in the ethereal layer. By this method a trace of the chlorophyll may be lost, but if the flavones and anthocyanins are not removed, the results when estimated colorimetrically would be far too high due to their presence. The addition of alkali later also greatly deepens the yellow color of the flavones which would tend to give too high results for the chlorophylls when estimated colorimetrically.

Tests made on a pure chlorophyll solution show that a one per cent. sodium carbonate solution will not remove any of the green pigments from an ether solution. Hence, any green pigments removed by the sodium carbonate solution are probably chlorophyll pigments which were already decomposed in the leaf.

The separatory funnel should not at any time be shaken because emulsions will form, the chlorophyll will go into the water in the colloidal state, and the separation will be more difficult. The wash water should remove none of the green or yellow pigments if the washing is properly done. The ether solution of the four pigments is now poured into a 250 cc. bottle. To this are added from 10–25 cc. of CH_3OH which has been saturated with KOH; this solution should have a clear color without a trace of yellow. It is shaken thoroughly and then set aside in the ice box till the next day when the chlorophyll will all be saponified and separated as a layer in the bottom of the bottle; the upper ethereal layer will contain the yellow pigments.

If it is necessary to proceed at once with the fractionation of the components, the saponification may be carried out by thoroughly shaking for 15 minutes or more. In this case there is danger that traces of the chlorophyll will not be saponified. The unsaponified portion will later be found in the xanthophyll fraction thus causing untrustworthy results to be obtained.

The alkaline solution of chlorophyllin salts, carotin and xanthophyll is now poured into a separatory funnel. Wash the bottle several times with 10–25 cc. of distilled water and add to the separatory funnel. Then wash the bottle with 25–50 cc. of ether to remove any yellow pigments. Add about 100 cc. or more of ether and shake strongly. Let stand 15 to 30 minutes and a yellowish ethereal layer of carotin and xanthophyll separates above, while the chlorophyllin salts separate as a greenish layer below. Run off the greenish layer and wash once or twice to remove any chlorophyllin salts which tend to remain in the ether layer with the yellow pigments; then add water to make about 75 cc. and extract the chlorophyllin solution with about 100 cc. of ether to remove traces of yellow pigments. Do this a second time if necessary, when the ether will be colorless or only slightly colored yellow. The greenish chlorophyllin salts are now run off into a 100 cc. volumetric flask. The ether solutions are combined and washed once with dilute potassium hydroxide solution (10–25 cc.) and twice with distilled water (10–25 cc.) to remove traces of alkali and chlorophyllin salts. These combined chlorophyllin solutions are made up to a volume of 100 cc. with water and the chlorophyllin salts are then estimated according to a method described in another paper (3) The

chlorophyllin solution may be made up to a larger volume if it is more convenient for the worker. Store the chlorophyllin solutions in an ice box till they are estimated.

The combined ether extracts are now evaporated in a flask at 50° C. or lower, to a few cc. and then evaporated strongly (using suction) to remove all traces of ether.

The residue is transferred by means of about 110 cc. of fresh petroleum ether and about 50 cc. of 85 per cent. methyl alcohol (made by making up 85 cc. of absolute CH_3OH to a volume of 100 cc. with water) to a separating funnel. Shake, let stand till the layers fully separate, and run off the methyl alcohol layer. From this petroleum ether solution the xanthophyll is further extracted by repeated shaking with methyl alcohol as follows: (1) two extractions with 25 cc. of 85 per cent.; (2) one extraction with 25 cc. of 90 per cent.; (3) two extractions with 92 per cent., using about 25 cc. for each extraction. Usually the last extraction is not colorless, and so further extractions with 92 per cent. methyl alcohol are made. Let the methyl alcohol and petroleum ether solution stand for about 5 minutes after each shaking for the layers to fully separate. In this way by means of the methyl alcohol the orange yellow xanthophyll is separated from the orange colored carotin which remains in the petroleum ether layer.

To the combined methyl alcohol xanthophyll extractions add 150–200 cc. or more of ether and pour the whole into a large separatory funnel. Now add distilled water equal in volume to the volume of methyl alcohol from which the xanthophyll is to be separated, shake vigorously and allow to stand about 15 minutes, when the xanthophyll will have collected in the upper ether layer, with a colorless aqueous methyl alcohol layer below. Treat, if necessary, with 10 cc. or more of a saturated solution of Na_2SO_4 or NaCl[3] to break emulsions, which, if not broken up, will make the separation of xanthophyll incomplete and very unsatisfactory. Run off the aqueous methyl alcohol solution and extract it a second time with ether. Now wash the combined ether solution once or twice with distilled water to remove traces of the salt and methyl alcohol. Run the ether solution of xanthophyll into a flask and evaporate at low temperature (using suction) to about 50 cc. Pour into a separatory funnel, add ether, and then a little water, to separate any methyl alcohol from the ether solution. Run off the lower layer, wash with water, and let stand a moment till most of the free water separates. Make up to 100 cc. in a volumetric flask and then estimate the pigment colorimetrically, or spectrophotometrically if very accurate results are desired (**2**).

[3] STECHE (**4**) recommends KCl and $CaCl_2$ for breaking up colloidal formations. These salts should be tried.

If the ether solution is not clear, it may be cleared by adding a few drops of absolute ethyl alcohol or by allowing it to stand for a few hours, by which time the water will settle out; or one may dry it by filtering through a layer of anhydrous sodium sulphate.

The petroleum ether solution of carotin is washed two or three times with distilled water to remove traces of methyl alcohol. The solution of carotin is then allowed to stand till the free water separates or is dried by filtering through anhydrous sodium sulphate and then run into a 100 cc. volumetric flask, cleared, made up to 100 cc. and is then estimated (**1**).

The following chemical changes take place in the method of separating and estimating the leaf pigments here outlined.

According to WILLSTÄTTER no chemical changes take place in the yellow pigments by the method of separation. During the separation of the yellow pigments, the two chlorophylls are changed when alkali is added as is shown in the following reactions:

$$C_{31}H_{29}N_3Mg\ (NH.CO)\ (COOCH_3)\ (COOC_{20}H_{39}) + KOH \rightarrow C_{31}H_{29}N_3Mg\ (NH.CO)\ (COOH)\ (COOH)$$

Chlorophyll α — Chlorophyllin α

$$C_{32}H_{28}O_2N_4Mg\ (COOCH_3)\ (COOC_{20}H_{39}) + KOH \rightarrow C_{32}H_{28}O_2N_4Mg\ (COOH)\ (COOH)$$

Chlorophyll β — Chlorophyllin β

Since working out the above method of determining chlorophyll and the yellow pigments in leaves a paper by STECHE (**4**) has come to the writer's attention. His recommendations are similar to those the writer has incorporated in the method as given in this paper. He omits the preliminary extraction because the flavones are later removed by washing from the ethereal solution of the chloroplast pigments. The writer has used a solution of sodium carbonate to remove the flavones and anthocyanins. He saponifies the chlorophyll in three steps using 5 cc. of saturated methyl alcoholic potash each time. His third change in WILLSTÄTTER's method concerns washing the acetone from the ethereal solution of chloroplast pigments. Instead of distilled water he uses a solution of sodium chloride, or, better, potassium chloride. He washes the ethereal solution of chloroplast pigments 5 times, each time with 100 cc. of 1 per cent. KCl solution, and then once with 100 cc. of 3 per cent. KCl solution. This step is similar to the writer's wherein NaCl solution is recommended. Any one interested in determining chlorophyll should read STECHE's paper.

Summary

1. A method is described for quantitatively extracting the chloroplast pigments from leaf tissues.

2. The chloroplast pigments after being freed from accompanying materials are then quantitatively separated into carotin, xanthophyll, and chlorophyll ($\alpha + \beta$).

3. These pigments are then quantitatively determined by methods published by the author.

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WASHINGTON, D. C.

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FURTHER EVIDENCE OF THE ESSENTIAL NATURE OF ZINC FOR THE GROWTH OF HIGHER GREEN PLANTS

ANNA L. SOMMER

(WITH THREE FIGURES)

Very little has been done to demonstrate the essential nature of zinc for the growth of higher green plants. Although MAZÉ, as early as 1914 (**1**), showed it to be essential for the normal development of maize, no further work seems to have appeared until the writer showed that zinc as well as boron (**2**) is essential for the normal growth of barley and sunflowers.

It is not evident that a given element which is essential in only small quantities for plant growth may be equally necessary for all groups of flowering plants. It is therefore desirable to extend the range of experimental material, which in the case of zinc has embraced only two representatives of the grasses and one of the Compositae. We now have plants of two other families to add to the list.

The technique used in this investigation was the same as that described in the paper reporting the work on barley and sunflowers. All salts were especially purified, the solution culture method was employed, and the plants grown in pyrex containers. Precautions were taken to prevent contamination of the solutions by dust.

The solutions had the following composition:

	Per liter		Per liter
KNO_3	0.80 gm.	Mn (as $MnSO_4$)	0.0015 gm.
KH_2PO_4	0.15 gm.	Al (as $Al_2(SO_4)_3$)	0.0005 gm.
$MgSO_4 \cdot 7H_2O$	0.50 gm.	NaCl	0.0127 gm.
$CaSO_4$, saturated solution	300 cc.	Cu (as $CuSO_4$)	0.000125 gm.
B (as H_3BO_3)	0.0005 gm.	I (as KI)	0.00025 gm.
		F (as KF)	0.00025 gm.

Iron as $FeSO_4$ was added when needed. An excess of silicon as SiO_2 was added to the solutions. The control cultures received in addition 0.0005 grams per liter of zinc as $ZnSO_4$.

To add to the families of plants already investigated, the Polygonaceae, represented by buckwheat, and the Leguminosae, represented by Windsor beans, *Vicia Faba,* and red kidney beans were studied.

Buckwheat responded to the lack of zinc in a way similar to that of the sunflowers; that is, the plants without zinc grew more slowly than the controls even during the early stages of development. None of the buckwheat plants, however, died before reaching the flowering stage. At maturity, plants grown without zinc were much smaller than the controls. The aver-

age dry weight per culture of buckwheat grown without zinc was 1.94 grams, that of the controls was 8.45 grams. Ten cultures of four plants each were included in each group. Buckwheat plants grown with and without zinc are shown in figure 1.

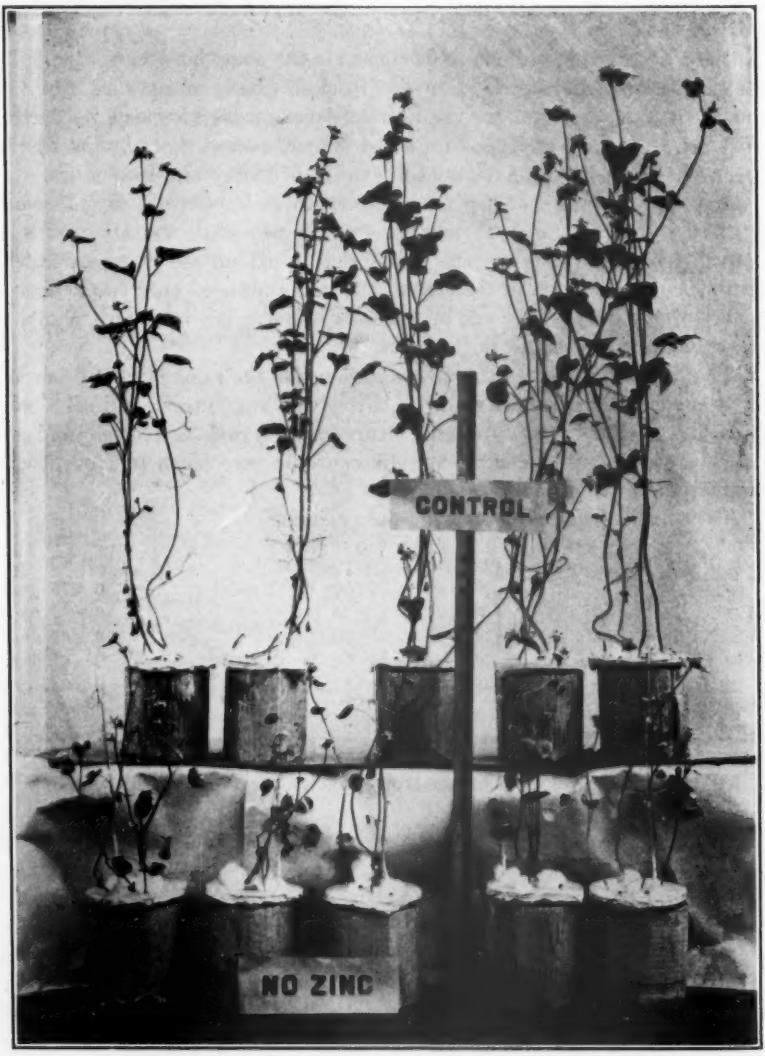

FIG. 1. Buckwheat grown with and without zinc.

The response of the legumes was quite different. In the case of the Windsor bean there was no visible difference between plants grown with and without zinc until the flowering stage was reached. At this stage there was a sudden and rapid abscission of the leaves. Most of the flower buds fell off and in the very few cases in which seed pods appeared, no seeds were formed. The average dry weight per culture of Windsor beans grown without zinc was 13.3 grams, that of cultures with zinc was 27.0 grams. The plants in culture solutions with zinc were growing well, blooming freely, and producing seeds when harvested. The plants without zinc were in very poor condition. Ten cultures of four plants each were included in each group. Windsor beans grown with and without zinc are shown in figure 2.

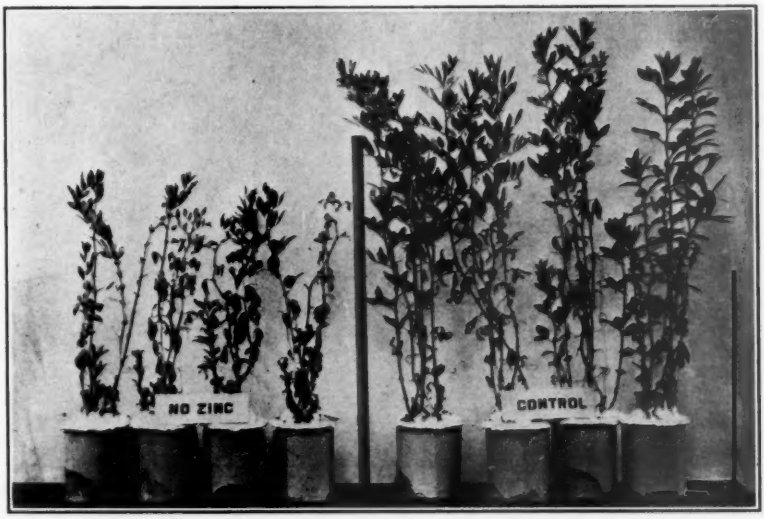

FIG. 2. Windsor beans grown with and without zinc.

The results obtained with red kidney beans were very similar to those obtained with the Windsor beans. The same number of cultures and plants per culture were employed. Red kidney beans grown with and without zinc are shown in figure 3.

The difference in the response of different plants to the absence of the same element is a very interesting and probably important study. In the case of zinc, the few plants studied by the writer presented three different types of response. In the case of wheat and barley, the plants appeared

normal for about two weeks, the tops then stopped growing and gradually dried up, all plants without zinc dying in the early stages of development. In the second type of response, that shown by sunflowers and buckwheat, plants showed the effects of the lack of zinc very soon after being transferred to zinc free solution. They made very poor growth but lived much longer than the barley, and, in the case of the buckwheat, produced a few poor seeds. The third type of response displayed by the legumes is interesting not only because the effects of the absence of zinc was not shown until the flowering stage was reached but also because of the apparent suddenness and the severity of the injury. The mature buckwheat plants and

FIG. 3. Red kidney beans grown with and without zinc.

the sunflowers which survived gave the appearance of plants which had been grown in poor soil or with insufficient moisture while the legumes looked like badly diseased plants very soon after the first symptoms of injury from the lack of zinc appeared. It seems to the writer that these differences are too great to be accounted for by the difference in the amounts of zinc that may be stored in the seeds and that they indicate that there must be significant physiological differences among the higher green plants. Observations on plants grown with and without boron strengthen this conviction. Most of the fairly large number of dicotyledonous plants studied by the author make little or no growth in the absence of boron and,

where some growth is made, the roots are the first to show the injury. On the other hand several members of the grass family make considerable growth and the tops are the first to show injury.

Earlier work on essential elements did not show zinc to be necessary because of the lack of refinement of methods, the importance of which I have discussed in another paper (**3**). The fact that plants of five different families require zinc for normal growth makes it probable that this element is indispensable for all of the higher green plants. When we consider the work of mycologists who, under less carefully controlled conditions, have shown it to be so beneficial to certain of the fungi, we may expect that future investigations will place it in the list of elements essential to the whole of the vegetable kingdom.

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A STERILE CHARACTER IN SOYBEANS[1]

F. V. Owen

(WITH TWO FIGURES)

In 1924 a progeny of Manchu soybeans was found to be segregating for sterility. The segregation has since proved to be very clear-cut and a single factor mutation is held to be responsible for the results. Careful records had also been taken of the parental plants for two previous generations so it is quite certain that the origin was by means of a genuine mutation and not merely a heterozygous selection.

The following results were obtained by growing seed from the plant (No. 26–5–11) which bore the first sterile seeds.

	Normal plants	Sterile plants
Observed	83	28
Calculated	83.2	27.8

The segregation of the progeny happened to be almost perfectly in line with expectation. Approximately two-thirds of the normal segregates also proved to be heterozygous for the sterility factor in the next generation so there is little doubt about the mode of inheritance.

The exact cause of this sterility has not been determined but apparently the ovule is non-functional as well as the pollen grains. At first it was hoped that the sterility factor had effected a wholesale emasculation for convenience in hybridization, but all efforts to produce seeds on sterile plants have failed. Pollen grains are formed which appear to be normal until the anthers are about to dehisce, but at this time they take on a shrunken appearance which makes it very easy to classify normal and sterile plants microscopically. Heterozygous plants appeared perfectly normal in all respects.

The physiology of sterile plants

The behavior of the sterile plants after flowering is of special interest because growth in soybeans is largely determinate. After the first flowers drop from the sterile individuals there is frequently a second flowering and small pods are sometimes formed, but no ovules have ever developed and the sterility has been complete in every case.

These sterile plants are perfectly normal during the early stages of growth but after flowering time there are noticeable differences between normal and sterile plants. Sterile plants take on a much darker green

[1] From the Department of Genetics, paper no. 90 Wisconsin Agricultural Experiment Station. Published with the approval of the director of the station.

color, the leaves thicken, and the stems sometimes become greatly enlarged. Furthermore, the leaves on these plants remain green and firmly attached to the stem long after normal segregates have matured. Fig. 1 shows the characteristic form of a sterile plant, and fig. 2 the contrasting normal individual.

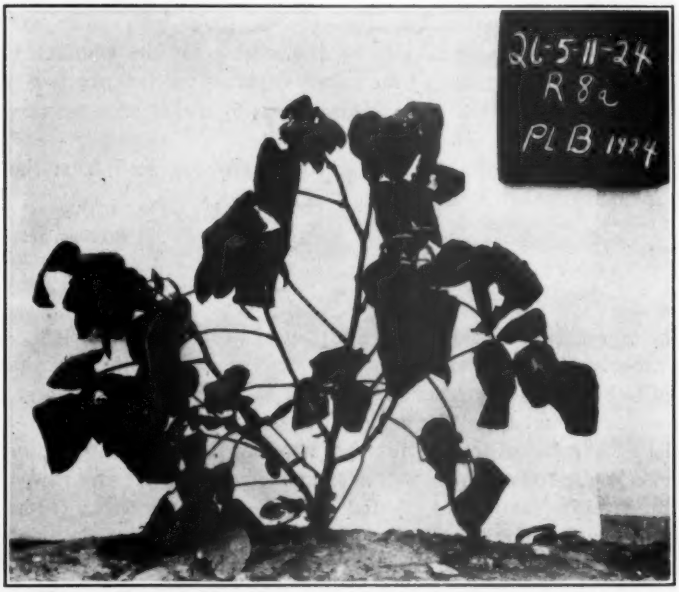

FIG. 1. Where can this plant store its soybeans? The chief significance of this sterile character lies in the nature of growth after flowering time. The plant still elaborates food material, but the determinate type of growth prevents the formation of new shoots. With storage channels all cut off, this "manufacturing establishment" becomes saturated with the very materials which it was designed to produce. There is a single factor difference between sterile and non-sterile plants.

No test of starch has been secured in the stems of normal plants after maturity but the iodine test has indicated the presence of considerable starch in the stems of sterile plants at the end of the season. This condition is to be expected because these sterile plants must be synthesizing considerable food material all the time, but the parenchymatous cells of the leaves and stem seem to constitute the only storehouse available. The whole situation, therefore, is quite like a modern canning factory that runs short of the usual tin cans. The section of the factory where raw materials are taken in may work ever so efficiently at first but it soon becomes congested from lack of storage containers.

The peculiar behavior of sterile plants, naturally deprived of a storehouse for elaborated food material, has also been of interest in connection with a study of mottling of the seed coat because of a possible connection between the two phenomena. In previous studies (**2**) abnormal plants have been described which resembled sterile plants very much in that the leaves thickened and turned a dark green color. These abnormal plants developed an extreme amount of mottling in the seed coat, and it is assumed that the excess accumulation of elaborated food material was associated with the development of glucosidal pigments.

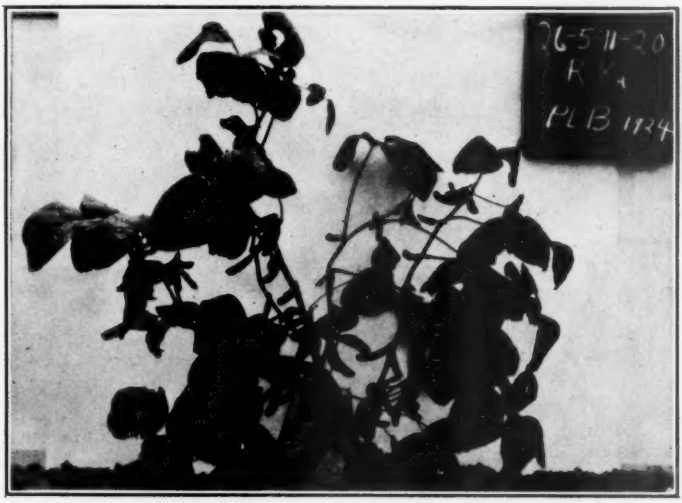

FIG. 2. Normal plant with plenty of pods for storage space. The seeds of normal plants make a natural storehouse for elaborated food material. It is believed that this is a very important factor in determining the ultimate efficiency of normal thrifty plants. This normal plant is a sib to the sterile individual shown in fig. 1.

It is hoped that a more thorough knowledge of the physiological peculiarities of sterile plants of this sort may sometime be available. MURNEEK's (**1**) interpretation of the stimulus given to a plant by setting fruit is very interesting and it seems natural to suppose that a plant should be at its maximum metabolic efficiency after a full growth of foliage has become established, provided that good storage channels are available. It is likely that a more careful study of plants entirely deprived of reproductive organs might also be pursued with profit. The soybean should be particularly well adapted for such studies because of the determinate type of growth.

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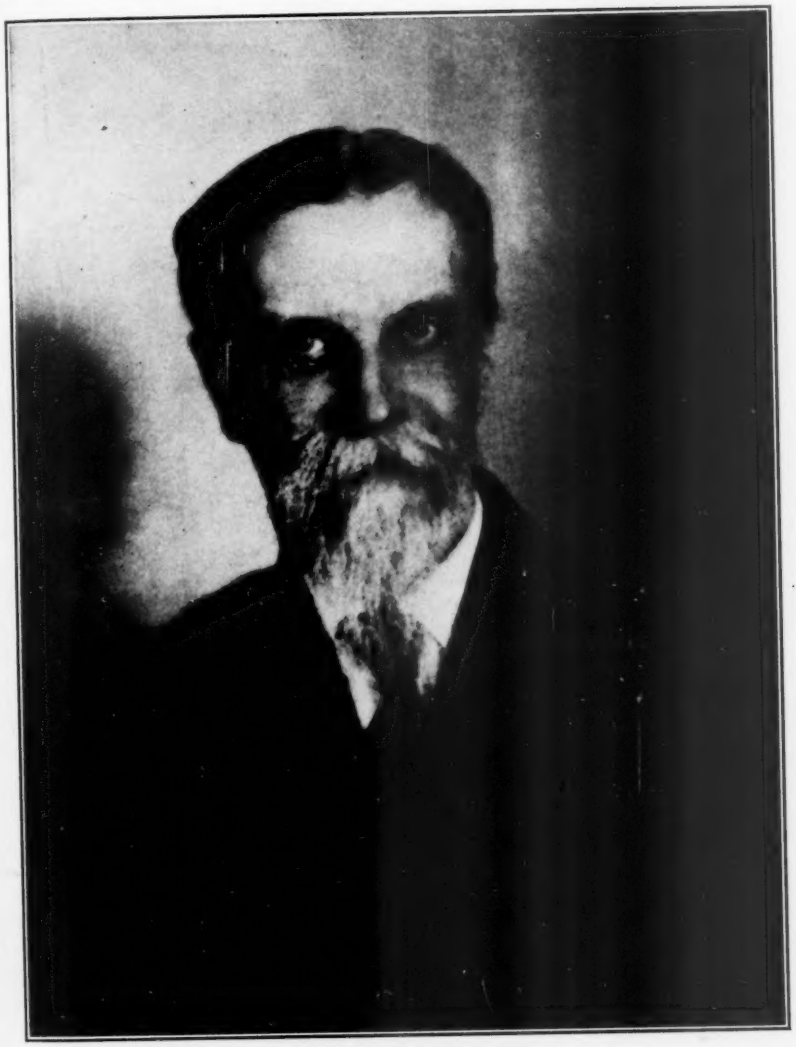

CLEMENT ARKADEVICH TIMIRIAZEFF

UNIV. OF MICH.

BRIEF PAPER

TIMIRIAZEFF AND THE TIMIRIAZEFF AGRICULTURAL INSTITUTE

(WITH ONE PLATE AND TWO FIGURES)

The devotion of CLEMENT ARKADEVICH TIMIRIAZEFF to the welfare of the Russian common peoples and his staunch advocacy of intellectual freedom in the universities of old Russia has placed him in the front rank of the leaders in the revival of new Russia. Much of the good in the new régime is the work of TIMIRIAZEFF and his associates, and he lived to see the overthrow of the Tsar's régime and the adoption of part of the ideas which he advocated, yet not to see the later evils of the revolution, since he died in 1920. Plate VI is from a portrait of the great plant physiologist.

Born on May 23, 1843, of an old and noble family at Leningrad TIMIRIAZEFF's life covered a period of active change of ideas in the biological sciences, and in this change he took an active part. He was responsible for the introduction of many of the newer ideas in biology and agriculture in Russia.

TIMIRIAZEFF's education at home, according to the Russian custom, equipped him with a thorough knowledge of languages and with a fundamental training for the University. He matriculated under the science faculty of the University of Leningrad in 1861. He passed his license examination five years later and was awarded a medal of gold for the excellence of his paper on the Liverworts. Following his first University degree he published a number of scientific papers and also took part in the political and literary developments of that day.

In 1868 appeared his first paper on photosynthesis. Most of the writings of TIMIRIAZEFF have been upon questions concerned with the chemistry and physics of carbon assimilation. He went into problems in the most minute detail, yet never lost sight of the fundamental principles underlying the research. It was this happy combination of minute exactness in detail and broad perspective of the whole of the research, which he instilled into his students with rare ability. TIMIRIAZEFF as a teacher had a remarkable faculty of imparting his great fund of scientific and historical information to his students as well as of creating in his class rooms an atmosphere of the highest culture. His lectures were characterized by clearness, and his demonstrations by simplicity.

TIMIRIAZEFF's advanced training was acquired at Heidelberg where he worked under BUNSEN, KIRCHOFF, and HOFMEISTER, and took a course under

HELMHOLTZ. Training in this exacting school left its impress upon all his experimentation. But it was at Paris that he received his inspiration under BOUSSINGAULT, and it was to BOUSSINGAULT that he frequently returned after his establishment as professor at Moscow. The connection between his experiments and the teachings of BOUSSINGAULT is not hard to trace. TIMIRIAZEFF published a memoir of BOUSSINGAULT in 1887. While still a student at Paris he heard BERTHELOT lecture on thermo-chemistry and frequently afterwards renewed his friendship on visits to Paris.

On his return from abroad he was elevated to the rank of professor by the Moscow Academy of Agriculture. He started at that institution courses in all the branches of botany. In 1877 he was appointed professor of anatomy and plant physiology at the University of Moscow.

TIMIRIAZEFF applied himself to the solution of the agronomic problems of Russia, particularly to the maintenance of soil fertility and to mineral nutrition, two phases of agriculture which are so prominently developed in Russia today. He established at the Agricultural Academy a laboratory for the study of mineral nutrition in 1872. His book "L'Agronomie et la Physiologie Végétale" did much to establish the practical applications of his physiological researches.

The Academy was closed by the government in 1892 under the suspicion of sedition and on account of sympathy with the movements of the agricultural classes which had been a continual source of trouble for the Tsar's government since 1872. The appointment of TIMIRIAZEFF was withdrawn. The following year he occupied himself in the organization of the Congress of Naturalists at Moscow, of which he was elected president. In the years next following, although deprived of his university connections, he continued publication on agronomic subjects and also devoted his energies to the establishment of the Darwinian theory in Russia. During these years he did much to popularize natural science.

He was reestablished in the chair of Anatomy and Plant Physiology at Moscow University in 1901, but resigned in the following year to devote his time to the botanical laboratory. In 1903 he gave the Croonian lecture before the Royal Society of London, summarizing in a clear manner the results of his thirty-five years of research under the title "The Cosmical Functions of the Green Plant." He was honored with the degree of LL.D. by the University of Glasgow in 1901 and received the honorary D. Sc. from the Universities of Geneva, and of Cambridge and Oxford. In 1911 he again resigned from the faculty at Moscow University as a result of the conflict of a number of the university professors with the ministry of public instruction.

From this time on he was unable to undertake exhausting tasks on account of ill health. On the occasion of his seventieth birthday he declined a public celebration in his honor on account of failing health.

TIMIRIAZEFF was a strong advocate of the freedom of thought in the university. This gained for him the following of the more venturesome spirits of the age in which he lived. When this group came into power after the revolution it perpetuated his memory in a manner eminently fitting. The Agricultural Institute in the suburbs of Moscow was named in recognition of his influence in the field of Agriculture.

This academy was founded in 1872 as the first Russian Agricultural Higher School (Petrovskoe-Rasumovskaya Academia) in the grounds of the castle built in 1678 as a residence for the future Tsar Peter I. The college buildings have been repaired and new laboratories for chemistry and

FIG. 1. Library and Administration Building before the park, and to the right, the Klub, a former church at the TIMIRIAZEFF Agricultural Institute, Moscow. Photo by HARVEY.

dairying erected since the revolution. The Academy provides for the instruction of more than three thousand students. The laboratory of plant physiology has been disorganized in the last two years since no appointment has been made to the vacant professorship. The mineral nutrition work of DR. PRIANISHNIKOV occupied five large and well equipped laboratories and had extensive greenhouse space. Interesting work was in progress on root excretion and upon sterile water cultures. There are eight major buildings at the Academy including a good library (see fig. 1). There are extensive plots for plant breeding. The old church of the Tsar, beautifully built in the old Russian style, has been transformed into a social club.

FIG. 2. Statue of TIMIRIAZEFF at TIMIRIAZEFF Circle in Moscow. Photo by HARVEY.

At the TIMIRIAZEFF Circle at Moscow there was erected in 1921 a statue of TIMIRIAZEFF in gray granite, the face an excellent portrait, giving evidence of the nobility of his character. (See fig. 2).—R. B. HARVEY, *Cambridge University, England.*

NOTES

Institute of Chemistry.—The second Institute of Chemistry will be held at Northwestern University, Evanston, Illinois, from July 23 to August 18, 1928. Dr. HARRY N. HOLMES, of Oberlin College, will occupy one week in the discussion of colloids, and Prof. WHITMORE, of Northwestern, will lecture on modern organic chemistry. Other lectures will be given by Dr. B. S. HOPKINS, of Illinois, on Inorganic Chemistry; by Dr. A. I. KENDALL, of the Northwestern University Medical School, on the Chemistry of Bacteria; and by Dr. VICTOR K. LA MER, of Columbia University, who will discuss the modern developments in Physical Chemistry.

The industrial chemistry will be of minor interest to plant physiologists. This phase of modern chemistry will be discussed by Dr. W. T. READ, of the Texas Technological College, and Dr. GERALD L. WENDT, of the Battelle Memorial Institute, who will consider particularly industrial research as related to modern industry.

The Chemical Institute held at Pennsylvania State College last year was a great success—not financially, but in what it accomplished. The second Institute will be worth attending by those who desire to keep abreast of the modern trends of fundamental chemical research.

Colloid Symposium.—One of the valuable features of the sixth National Colloid Symposium at the University of Toronto is an exhibition of experimental methods in colloidal work. Such displays play an important part in the rapid development of scientific research, and with the wide interest now shown in colloidal phenomena, this exhibition will no doubt be a very valuable feature of the sixth symposium.

Summer Meeting.—The Purdue Section of the American Society of Plant Physiologists some time ago extended an invitation to the Society to hold a summer meeting at Purdue University during the late summer or early autumn of 1928. The date suggested is September 4–5, and the Executive Committee has voted to accept the invitation. Previous summer meetings have been very successful, and the meeting at Purdue will be of more than usual interest. More details as to the time of meeting will be given in the July number of PLANT PHYSIOLOGY.

Dr. Patrick J. O'Gara.—The American Society of Plant Physiologists records with regret the first loss it has sustained by death of a member. Dr. PATRICK J. O'GARA, formerly connected with the investigations in the Rogue River Valley, Oregon, and for many years director of the agricultural and smelter byproducts investigations of the American Smelting and Refining Co., at Salt Lake City, Utah, died on September 17, 1927, at the age of 55 years. His work was mainly in the field of plant pathology and plant physiology, with special reference to the effects of sulphur dioxide and other smelter wastes on agricultural plants. Appropriate resolutions were drawn up at the Nashville meeting, and made a part of the permanent records of the Society.

Stephen Hales Prize.—The committee on the award of the first STEPHEN HALES Prize at the fifth annual meeting in New York, has recently been appointed by the president of the Society. The chairman of the committee is Dr. JAMES BERTRAM OVERTON, of the University of Wisconsin, and the other members are Dr. C. R. BALL, of the U. S. Department of Agriculture, and Dr. A. L. BAKKE, of the Iowa State College. The committee is charged with the selection of a worthy recipient of the prize sometime prior to the New York meeting, and the announcement of the award will probably be made at the annual dinner for all plant physiologists.

The Membership List.—The members of the American Society of Plant Physiologists have received a copy of the membership list published by the Secretary some time ago. The steady growth of the Society is very gratifying. No doubt many of the members know of friends who should be invited to become active members of the Society. The development of the journal is going forward as rapidly as its support makes such a program possible. The library subscriptions have become a splendid source of support, and will no doubt increase in numbers with time. Each member should help to place PLANT PHYSIOLOGY in the library of the institution he serves, so that a permanent and complete file may be available at the institution library.

Purdue Section.—The Purdue Section is an active and vigorous unit of the American Society of Plant Physiologists. The president of the local organization is Prof. C. L. PORTER, who has taken an interest in the development of the Section ever since it was organized. The experience of the group at Purdue shows that these local groups can perform a splendid

service in the institutions where they are active. Other universities would no doubt profit by such sectional activities wherever there are enough members to permit the formation of a section.

Bibliography of Seed Germination.—The Committee for Publication and Registration of the International Seed Testing Association has compiled a "bibliography of germination of seed," which will prove to be unusually valuable to those interested in the physiology of germination and related problems.

The table of contents is published in six languages, English, German, French, Danish, Italian and Dutch. The main headings under which the literature is classified, are as follows: A. Influence of physical factors on germination; B. Influence of chemical factors on germination; C. Influence of living factors (parasites, microbes, injurious insects); D. Influence of state of development; E. Chemistry of germination; F. Technique of germination tests; G. Publications on germination without further indications; and I. Agricultural seeds. The list of authors contains the names of about 1700 investigators and the literature citations occupy 212 pages, including a supplementary list.

Under each main heading, the chronological order of citation is followed. The earliest papers go back to the earlier part of the 19th century, and the latest ones are dated 1928. The list is not without omissions, but is so large as to be extremely helpful to students of germination behavior.

The list is mimeographed, and should not be very expensive if it is planned to place it on sale. The chairman of the Committee is Dr. W. J. FRANCK, of Wageningen, Holland. The committee has performed a very useful service in preparing this extensive bibliography, and the service will be appreciated by all who have occasion to consult the literature of seed germination.

International Critical Tables.—The third volume of this monumental work has come from the press of the McGraw-Hill Book Co. The volume covers two general fields: the pressure-volume-temperature relations of one-phase systems (density, specific gravity, thermal expansion, and compressibility); and phase equilibria data. The first section occupies 199 pages, and considers, among other things, the pressure-volume-temperature relations of substances which are gases at 0° C. and one atmosphere pressure; nitrogen; thermal expansion of inorganic compounds in the liquid state; of liquid organic compounds; of aqueous solutions of inorganic substances and strong electrolytes; compressibility data for water, alcohols, crystals, pure metals; and density and specific gravity of water.

The second section contains vapor pressure data for a number of the elements, P, S, Se, Te, and the halogens, atmospheric gases, metals, chemical compounds in the crystalline state, ice and water, organic liquids, etc.

There are also tables of partial pressure, vapor pressure lowering, vapor pressure of acids in aqueous solution, and a number of tables devoted to boiling point data with non-volatile solutes. Solubility data are given for gases in liquids and in solutions, for liquids in liquids, and miscibility data with three components. There is also a section on the relative efficiency of drying agents.

The volume contains 444 pages of tables and other information, much of which is occasionally useful to investigators in the biological field. Every institutional library will want a complete set of these volumes.

Indicators.—Although FURMAN'S translation of KOLTHOFF'S work on Indicators has been available for about two years, attention is called to it because of its usefulness in connection with the theory of indicator action. There are eight chapters in the volume, as follows: Neutralization; amphoteric compounds; the color change of indicators; the use of indicators in quantitative neutralizations; the colorimetric determination of hydrogen-ion concentration; practical applications of the colorimetric determination of hydrogen-ion concentration; indicator papers; and theory of indicators. Several useful tables are found at the back of the book, the dissociation constants of water at different temperatures, average degree of dissociation of salts at 18° C., dissociation constants of important acids and bases, and the transition range of indicators.

The book is a valuable one and belongs on the convenient book shelf. It is published by Wiley, 1926, and the cost is $3.50.

Potentiometric Titrations.—This book also is a translation of Dr. I. M. KOLTHOFF'S work by Prof. N. HOWELL FURMAN, of Princeton University. The first six chapters, Part I, treat the fundamental principles of potentiometric work. The chapters bear the following headings: Principles of precipitation and neutralization reactions; electrode potentials, oxidation and reduction; oxidation and reduction reactions and their equilibrium constants; relation between the change of the ion concentrations and electrode potential in combinations of ions; the relation between change in the ratio oxidant: reductant and the electrode potential in oxidation-reduction reactions; and general conditions for the performance of potentiometric titrations.

Part II contains five chapters. The first two of these deal with general matters connected with the technique of potentiometric titrations, and the detection of end points of potentiometric determinations. The last three chapters are devoted to precipitation and complex-formation with metallic or non-metallic electrodes, such as silver, mercury, copper and iodine; neutralizations; and oxidation-reduction reactions.

Sixteen pages of citations, and seven tables, complete the work. It is a companion volume to the one on Indicators, and with the latter forms a splendid two volume work. The price of Potentiometric Titrations is $4.50, and the publishers are Wiley and Sons, New York.